Expression and Regulation of ABC Transporter Genes during Liver Regeneration

RIJKSUNIVERSITEIT GRONINGEN

Expression and Regulation of ABC Transporter Genes during Liver Regeneration

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Cover

Impresssion of the hepatic microsecretory unit (Introduction: Figure 1.3)

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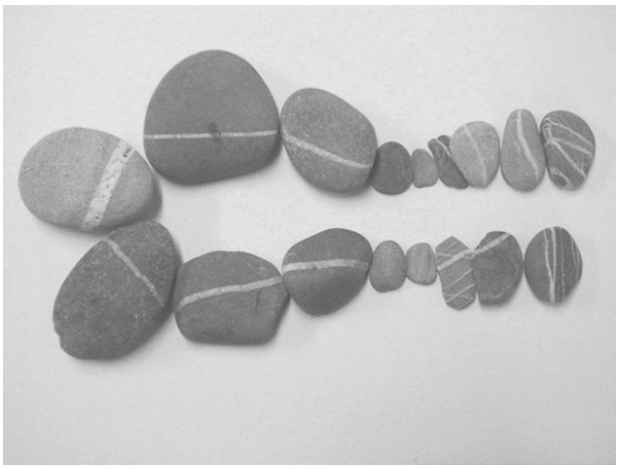

1.1 Introduction

The liver has various important physiological functions, including the metabolism of (potentially) toxic endo- and xenobiotics. Metabolites are secreted by liver cells into blood or into bile for removal from the body via urine or faeces, respectively. ATP-binding cassette (ABC) transporters have an essential role in this transport function of liver cells.

Although it is a highly differentiated organ, the liver retains the ability to regenerate after loss of tissue. This process involves active cell division. Tissue deficits can occur after surgical removal of a part of the liver, e.g., for treatment of hepatic carcinoma, or can be caused by loss of functional cell mass due to the effects of toxic chemicals or viruses. Under such circumstances the remaining cells are able to proliferate to make up for the loss of tissue. Regeneration of the liver can occur through proliferation of mature hepatocytes or, when the hepatocytic cell cycle is blocked, through activation of hepatic progenitor cells that subsequently develop into functional liver cells. In addition, bone marrow-derived cells may migrate to the liver and repopulate the diseased organ to a certain extent.[1,2]

Cells regenerating the liver must be well protected against toxic metabolites and xenobiotics. ABC transporters may be essential for this protection. In this thesis the expression of ABC transporters in different models of liver regeneration in rats and in human liver disease is described. The signal transduction route involved in the regulation of Mdr1b expression has been studied in detail, as expression of Mdr1b, an ABC transporter located in the canalicular membrane of hepatocytes, is highly increased during liver regeneration.

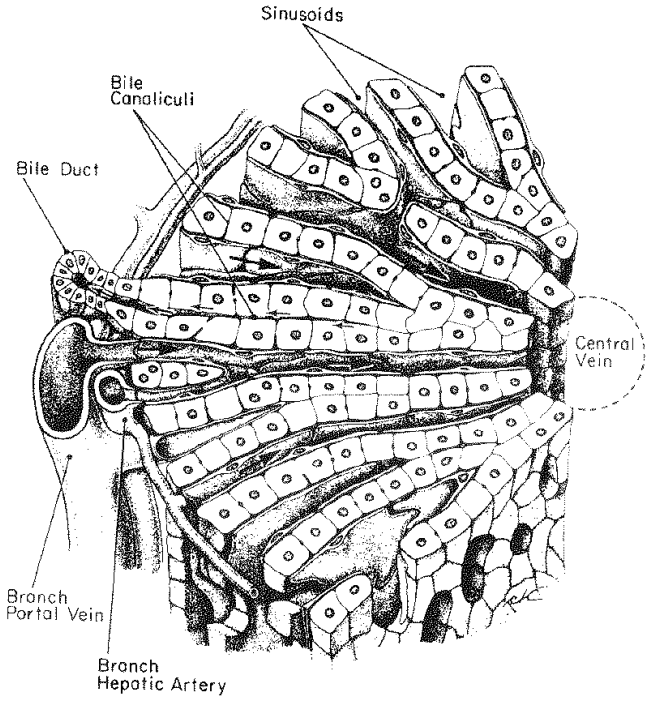

Figure 1.1: Schematic representation of a liver lobule. Large arrows indicate the flow of sinusoidal blood, originating from portal veins (carrying nutrients from the intestine) and hepatic arteries (providing oxygen); small arrows represent flow of bile towards the bile ducts. Reprinted from Bloom *et al.* (1975), A textbook of histology.

1.2 The liver

The liver consists of different cell types (Figure 1.1). Hepatocytes comprise ∼80% of total liver cell mass. Hepatocytes are polarized cells arranged in one cell layer-thick plates. The basal or sinusoidal membrane of hepatocytes contacts the space of Disse (see below). The apical or canalicular membranes of adjacent hepatocytes form the bile canaliculi. These connect with the terminal bile ductules via the canals of Hering. These canals are partially lined by hepatocytes and partially by biliary epithelium.[3,4] The ductules pass into the interlobular bile ducts. The cholangiocytes lining the bile ducts are heterogeneous in appearance. The bile ductules are lined by small cholangiocytes, which are cuboidal in form, whereas the larger bile ducts are lined by large cholangiocytes, which have a more columnar appearance.[5,6]

Hepatocytes, bile ductuli and bile ducts can be considered as a hepatic microsecretory unit (a schematic presentation of a hepatic microsecretory unit can be seen in Figure 1.3). The hepatocytes produce the primary bile. The main determinant of bile formation is

transport of bile salts, forming the "bile salt-dependent bile flow". Excretion of organic anions, especially of reduced glutathione, further contributes to bile flow and constitutes the "bile salt-independent bile flow".[7] Primary bile is modified in the bile ducts where the cholangiocytes add water, chloride, and bicarbonate to bile, but absorb glucose, amino acids and, to some extent, bile salts.[6]

The sinusoids contain endothelial cells, Kupffer cells, pit cells, and stellate cells. The endothelial cells are fenestrated and constitute the barrier between the hepatocytes and the blood stream, allowing exchange of fluids between the blood and the space of Disse, but hindering the passage of cells. Kupffer cells (macrophages) and pit cells (natural killer cells) have important roles in immune response and phagocytosis. The hepatic stellate cells, located in the space of Disse, contain vitamin A-rich lipid droplets. Beside storage of vitamin A, stellate cells play a major role in fibrogenesis.[8]

The liver has a dual blood supply. Oxygen-rich blood (25% of total volume) enters via the hepatic artery, whereas blood coming directly from the intestine enters via the portal vein (75%). Blood leaves the liver via the hepatic vein. A terminal hepatic artery, a terminal portal vein and a terminal bile duct form a so-called portal triad from which rows of hepatocytes radiate towards the hepatic veins. This forms the smallest functional unit in the liver, called the acinus. The hepatocytes in the acinus are functionally divergent, dependent on their localization. Zone 1 is formed by the periportal hepatocytes, and zone 3 by the perivenous hepatocytes. Bile flows through the canalicular network in the direction opposite to blood flow, i.e., from zone 3 to zone 1, and collects in the bile ducts (Figure 1.1).

Oxygen tension and the concentrations of metabolic substrates, cytokines, and hormones in the blood differ in the various zones. Consequently, various functions of the liver are located in specific zones. Hepatocytes in zone 1 have a higher capacity for detoxification of reactive oxygen intermediates by glutathione, glucose output, urea synthesis, and bile formation. The capacity for glucose uptake, glutamine formation and xenobiotic metabolism is higher in zone 3.[9]

1.3 Hepatic transport proteins

For uptake and secretion of compounds from and into the blood and bile, cells in the liver contain a large number of transporter proteins. Transport specificities, cellular localization and regulatory mechanisms of a number of these transporters are now known. A comprehensive picture is emerging, showing tight regulation of transporters and metabolic systems to maintain homeostasis. An example of this is the enterohepatic circulation of bile salts: bile salts secreted via the bile into the intestine are reabsorbed and returned to the liver to be absorbed by hepatocytes, where they actively suppress bile salt formation. Members of the nuclear receptor family, that are activated through binding of specific ligands are essential in this regulation.[10]

With the completion of the sequencing of the human genome, all transporter proteins can now be identified based on their sequence homology. Characterization of substrates

and determination of physiological functions for all transporters will be the next goal.

Hepatic uptake transporters

The uptake of compounds into hepatocytes and cholangiocytes is predominantly mediated by members of the solute carrier superfamily (SLC). Members of this family have diverse structures and may contain 7 to 12 transmembrane domains. Driving forces for transport differ between the transporters.

The main transporter for the uptake of bile salts from blood into hepatocytes is the sodium-dependent taurocholate co-transporting protein Ntcp/NTCP (gene symbol *Slc10a1*). Ntcp is located in the basolateral membrane. Its expression is not zonated.[11] Transport by Ntcp is driven by the co-transport of Na^+ ions. The Na^+-gradient required for this transport is generated by the Na^+/K^+ ATPase. Ntcp preferentially transports bile salts conjugated to glycine or taurine, e.g., taurocholate, and to a lesser extent unconjugated bile salts, like cholate, in co-transport with sodium (reviewed by Kullak-Ublick *et al.*[12]). Basal expression of Ntcp is controlled by members of the nuclear receptor family, namely by the retinoid X receptor (RXR) and retinoic acid receptor (RAR) heterodimer RXR:RAR.[13] High levels of bile salts activate the bile acid receptor FXR/BAR, which results in increased transcription of the short heterodimer partner-1 (Shp-1). Shp-1 in turn decreases the activity of RXR:RAR, resulting in decreased expression of Ntcp. This way, hepatocytes reduce the uptake of bile salts.[14]

In hepatocytes Oatp1 (*Slc21a1*), Oatp2 (*Slc21a5*), and Oatp4 (*Slc21a10*) contribute to the transport of a wide range of compounds, including organic anions (among which bile salts and glutathione conjugates) and large cations, although their individual transport characteristics differ slightly.[15–18] Oatp1 and Oatp2 are able to transport bi-directionally. Their driving force appears to be anion exchange. Both proteins can transport glutathione (GSH) and for Oatp1 the reverse transport of GSH may be an important driving force.[19,20] Oatp1, Oatp2, and Oatp4 are located in the basolateral membrane of hepatocytes. Oatp1 is homogenously expressed along the liver acinus, whereas Oatp2 and Oatp4 are predominantly localized in zone 3.[16–18,21–24] In humans, the OATP proteins in liver are OATP-C (OATP2, *SLC21A6*), which has the highest homology with rat Oatp4, OATP8 (*SLC21A8*), and OATP-B (*SLC21A9*). These transporters are all present in the basolateral membrane of hepatocytes.[25–27] In addition, OATP-A (OATP1, *SLC21A3*) is expressed at a low level in human liver.[28]

Small organic cations, including drugs, choline, and monoamine neurotransmitters, are transported by members of the organic cation transporter family. Until now only one member of this family, Oct-1 (*Slc22a1*), has been identified that is expressed at high levels in liver. It is located in the basolateral membrane of hepatocytes.[29]

In the liver, uptake transporters of bile salts have not only been identified in hepatocytes, but also in cholangiocytes. The sodium-dependent bile salt transporter Asbt (*Slc10a2*) is expressed in the apical membrane of large cholangiocytes, as well as in ileum and kidney.[30,31] A splice variant of Asbt, t-Asbt, has been identified in the basolateral membrane of large cholangiocytes, and may function as a bile salt exporter.[32] It has been suggested that Oatp3 (*Slc21a7*) may also mediate the uptake of bile salts by cholangio-

cytes. Its expression in normal liver is however very low,[24] and its exact role is currently unknown.

Bile salts that are absorbed by the large cholangiocytes subsequently recirculate to the hepatocyte sinusoidal membrane. This shunting of bile salts back and forth between hepatocytes and cholangiocytes has been termed cholehepatic shunting.[33] It may promote additional bile formation, but to what extent this occurs is not known.

Hepatic export transporters

Export of compounds from the liver is predominantly carried out by ABC transporters. The structure of these transporters is highly conserved. Most ABC transporters have two sets of 6 membrane spanning domains (MSD1 and MSD2) (Figure 1.2A), though some have an additional N-terminal extension with five transmembrane domains (MSD0) (Figure 1.2B). Two intracellular loops of approximately 300-400 amino acids (NBD1 and NBD2) contain Walker A and B motifs, which are involved in the binding and hydrolysis of ATP. This provides the energy needed for the transport against a concentration gradient.[34,35] Between the two Walker motifs a short, highly conserved, motif is found. This "signature sequence" or Walker C motif is a distinctive feature of all ABC transporter proteins.[34] A number of ABC transporters are half transporters, consisting of only six transmembrane domains and one intracellular ATP binding domain (Figure 1.2C). These proteins, however, most likely dimerize in the membrane to form a functional unit.

Based on their sequence homology, the members of the ABC superfamily have been divided into seven subclasses (A to G). ABC transporters are not exclusively located in the plasma membrane, but have also been localized to intracellular organelle membranes as the peroxisomes, mitochondria and the endoplasmic reticulum. In this introduction, focus will be on transporters located in the plasma membrane with specific attention to hepatic transporters. An extensive overview of all human ABC transporters can be found on http://nutrigene.4t.com/humanabc.htm.

The Abca family

The Abca family consists of 13 members. These transporters are characterized by an extra hydrophobic stretch of amino acids in the first intracellular loop (NBD1), putatively forming an extra loop in or through the membrane.[36] Members of the Abca transporter family are important for phospholipid transport. Best characterized is Abca1, which is expressed at high levels in fetal tissue where its expression correlates with apoptotic areas.[37] Mouse Abca1 has been shown to be involved in engulfment of apoptotic cells by macrophages.[37] In adult liver Abca1 may function as a phosphatidylserine transporter, involved in formation of pre-βHDL, allowing cholesterol uptake by apolipoprotein-A-I in high density lipoproteins.[38,39] Mutations in the human *ABCA1* gene have been linked to Tangier disease, a disorder characterized by the accumulation of cholesteryl ester in various tissues and absence of HDL in plasma.[40–42]

The Abcb family

Of the 11 Abcb family members, four are expressed in the cell membranes of hepatic

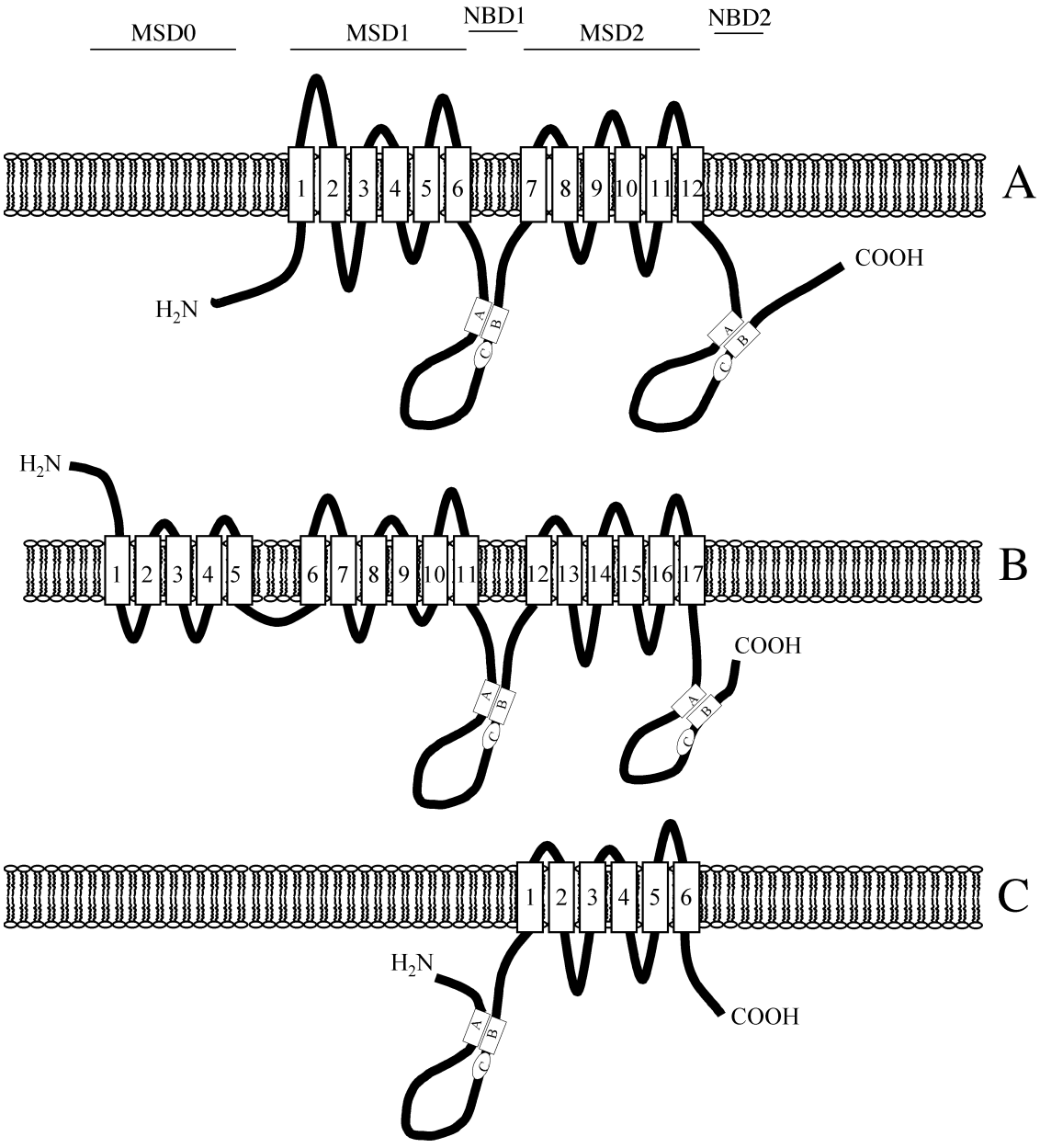

Figure 1.2: Membrane topology models of ABC transporters. Schematic representation of (A) the MSD1-NBD1-MSD2-NBD2 structure, (B) the MSD0-MSD1-NBD1-MSD2-NBD2 structure, and (C) the NBD-MSD structure of half transporters. MSD, membrane spanning domain; NBD, nucleotide binding domain.

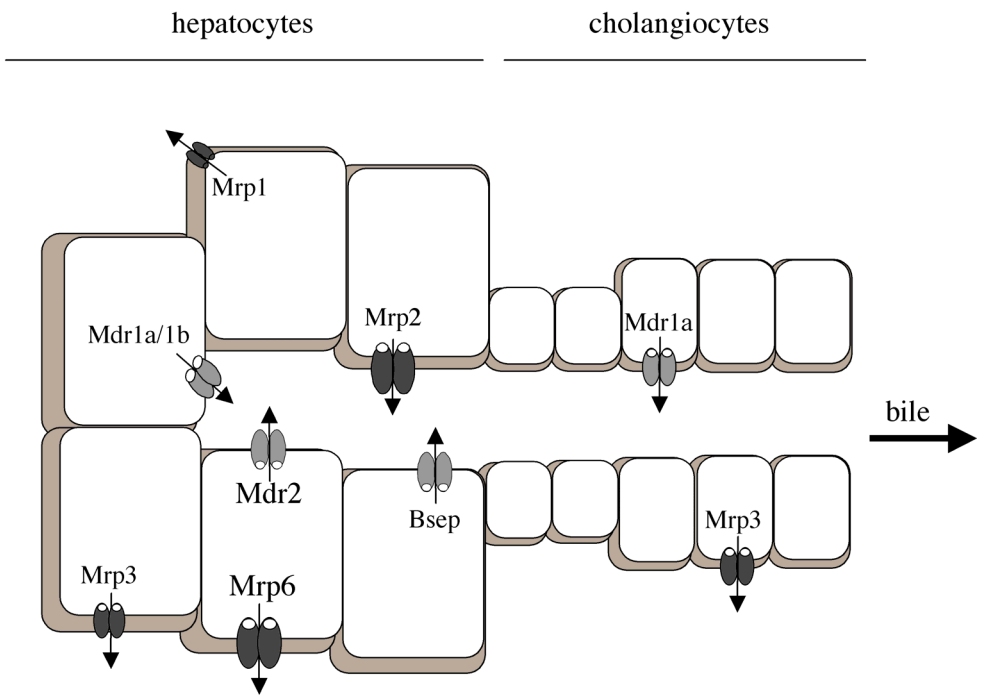

Figure 1.3: Schematic representation of ABC transporters in rat liver. Scheme of a hepatic microsecretory unit, depicting the localization of Abcb transporters (light grey) and Abcc transporters (dark grey) in hepatocytes and cholangiocytes.

cells, namely Mdr1a/b, Mdr2, and Bsep (Figure 1.3). The human multidrug resistance transporter MDR1 (*ABCB1*) has two homologues in rodents, i.e. Mdr1a/Mdr1b (*Abcb1a/Abcb1b*).[43,44] The proteins are expressed in the canalicular membrane of hepatocytes and the apical membrane of small biliary ductules in liver, as well as in other tissues with excretory functions, such as the kidney and the small intestine.[45] Mdr1 proteins are also expressed in bone marrow, fetal tissue, brain, and testis,[46–49] where they function in maintenance of the barrier function, by continuously removing hydrophobic compounds.[50–53] Substrates for Mdr1 proteins include structurally unrelated, mainly amphipathic cationic, compounds and small cations.[54–56] The expression of MDR1 in normal human liver is high compared to the expression of Mdr1a/1b in laboratory animals.[48,57,58]

Although not essential for basic physiological functions, MDR1 and Mdr1a/1b are essential for protection of the body from potentially toxic compounds. High expression of MDR1/Mdr1a/Mdr1b in tumor cells keeps the intracellular level of chemotherapeutics low. This causes multidrug resistance, the phenomenon that cells become resistant to various, structurally unrelated, chemotherapeutics.

The role of Mdr1 proteins in cellular protection may be complementary to the role of cytochrome P450 proteins: whereas Mdr1a/b protect the cell by transporting toxic compounds out of the cell, cytochrome P450 proteins can modify such compounds into more hydrophilic metabolites, allowing transport by different transporters. Studies in

Abcb1a/b nullizygous mice revealed an inverse relationship between Mdr1 protein expression and levels of cytochrome P450 proteins as CYP3a.[59] Besides its function as efflux pump for xenobiotics, additional physiological roles have been proposed for Mdr1. It may function as a transporter of endogenous compounds, such as steroid hormones, small peptides, or cytokines.[60–65] By transporting intracellular signal transduction factors, Mdr1 might influence signal transduction pathways. An anti-apoptotic role for Mdr1 has been proposed.[66–69] In these studies, Mdr1 function was impaired by inhibitors or antibodies. Alternatively, cells have been used that were induced to express Mdr1 protein either by drug selection or by retroviral transduction. As these interventions may have a large impact on the systems studied (discussed by Borst *et al.*,[70] and Johnstone *et al.*,[71]) the precise role of Mdr1 in the control of apoptosis remains to be elucidated.

In contrast to the drug-transporting Mdr1 proteins, Mdr2 (*Abcb4*, MDR3 in human) is a translocator of phosphatidylcholine.[72] Mdr2 is expressed at high levels in the canalicular membrane of hepatocytes, with a slightly higher expression in zone 1.[73–75] Its role was identified in *Abcb4* nullizygous mice that do not excrete phosphatidylcholine into their bile. As a result, bile salt micelles in primary bile are not masked by lipids and cause extensive damage to the hepatocytes and cholangiocytes.[72] Mutations in the human *ABCB4* gene are the underlying cause of progressive familiar intrahepatic cholestasis (PFIC) type 3. Patients present with increased cholestatic serum markers and high γ-glutamyltranspeptidase levels.[76]

Abcb11 encodes the bile salt export pump BSEP. This protein is a hepatocyte-specific transporter, present in high levels in the canalicular membrane.[77] Mutations in the human *ABCB11* gene are associated with progressive familiar intrahepatic cholestasis (PFIC) type 2, a disease characterized by low biliary bile salt concentration, elevated serum bile salt concentrations and normal γ-glutamyltranspeptidase levels.[78,79]

The Abcc family

The Abcc family consists of 12 members. At least four of these, Mrp1, Mrp2, Mrp3, and Mrp6, are expressed in healthy liver. *Abcc1* encodes Mrp1, expressed in the basolateral membrane of hepatocytes. Its expression in liver is low under normal conditions.[80,81] Mrp2 (*Abcc2*) is the apical isoform of Mrp1. Mrp2 is expressed in the canalicular membrane of hepatocytes,[82,83] as well as in kidney and intestine.[84,85] The substrate specificity of Mrp1 and Mrp2 is highly similar. Both are able to transport compounds conjugated to glutathione, glucuronate or sulfate. Physiological substrates include leukotriene C_4, bilirubin glucuronides, 17β-glucuronosyl estradiol, taurolithocholate 3-sulfate, and glutathione disulfide (GSSG). In addition, transport of other organic anions and several cationic drugs, requiring the presence of GSH, has been reported (reviewed by Renes *et al.*,[86] Keppler *et al.*,[87] and König *et al.*,[88]). As for Mdr1, over-expression of Mrp1 can confer multidrug resistance to tumor cells.[80] It has been demonstrated that Mrp2 also can confer resistance to cytotoxic drugs in *in vitro* systems,[89,90] but it remains to be shown whether this also occurs *in vivo*.

Mutations in the *Abcc2* gene causing premature stopcodons result in absence of Mrp2 protein in Transporter Deficient (TR^-) and in Eisai hyperbilirubinemic (EHBR) rats.[83,91]

These rats cannot secrete conjugated bilirubin. In humans, mutations in the *ABCC2* gene have been linked to Dubin-Johnson syndrome.[92,93]

Mrp3 (*Abcc3*) is localized to the basolateral membrane of hepatocytes and cholangiocytes. Mrp3 expression in hepatocytes is low in normal liver. It can only be detected in 1-3 layers of hepatocytes surrounding a hepatic venule. Mrp3 is, however, highly expressed in the basolateral membrane of cholangiocytes.[94] Mrp3 is also expressed in other tissues, including colon, small intestine, gallbladder, and at relatively low levels, in kidney.[95–97] Mrp3 is a transporter of glucuronide conjugates. In addition, Mrp3 is able to transport bile salts, with the highest affinity for taurolithocholate-3 sulfate, but Bsep substrates like taurocholate and glycocholate are also transported.[98] Glutathione conjugates are poor substrates for Mrp3.[99] The presence of Mrp3 in cholangiocytes suggests that it may have a role in the cholehepatic shunt of bile salts. In addition, Mrp3 may be responsible for the intestinal transport of organic anions. The hepatocellular expression of Mrp3 is highly increased in cholestatic conditions. As the expression of Mrp2 is down-regulated during cholestasis, Mrp3 may function to efflux compounds into blood that are normally transported by Mrp2 into bile.[98,99]

Mrp6 (*Abcc6*) is expressed at high levels in the liver. It is most likely localized in the basolateral membrane of hepatocytes, though apical staining has been observed as well. Thus far the only substrate for Mrp6 that has been identified is the cyclopentapeptide BQ123.[100] Deficiency of Mrp6 causes pseudoxanthoma elasticum, a systemic disease of elastic tissue, affecting the eyes, skin, and blood vessels.[101]

Recently, more members of the Abcc family have been identified. These include Mrp4 (*Abcc4*) and Mrp5 (*Abcc5*). Mrp4 and Mrp5 do not contain the MSD0 domain. They are nevertheless more related to other Mrps than they are to other ABC transporters.[102] Both transporters are ubiquitously expressed, although their expression in normal liver is relatively low.[95,102] Mrp4 and Mrp5 are organic anion pumps, but they are also capable of transporting cyclic nucleotides (cGMP and cAMP) and nucleotide analogues.[103–105] These transporters might therefore participate in signal transduction pathways in which cyclic nucleotides are involved. In addition, these transporters can cause resistance to nucleoside-based drugs that are clinically used in antiviral therapy.[103]

The cystic fybrosis transmembrane regulator (Cftr, *Abcc7*) is also a member of the Abcc family. Cftr presumably does not function as a transporter but as a Cl^- channel.[106] In the liver CFTR/Cftr is only expressed in the apical membrane of cholangiocytes.[107,108]

The Abcg family

The Abcg family consists of half transporters with an NBD-MSD structure, as depicted in Figure 1.2C.[109] This subfamily comprises eight proteins. Abcg1, g5, and g8 have been shown to be involved in the regulation of lipid-trafficking. Abcg1 is ubiquitously expressed.[110,111] Best characterized is its role in lipid homeostasis in macrophages.[110] Abcg5 and Abcg8 are expressed in small intestine and liver. These transporters have been implicated in the efflux of dietary sterols from the enterocytes back into the lumen and possibly from the liver into the bile.[112] Mutations in the human *ABCG5* and *ABCG8* genes have been linked to sitosterolemia, a disease in which affected individuals have increased levels

of plant sterols in plasma due to increased intestinal absorption.[113]

Bcrp (*Abcg2*) is highly expressed in placenta and at relatively low levels in many cell types, including the liver (apical membrane of hepatocytes) and the epithelium of the small intestine and colon.[114] It was recently demonstrated in mice that Bcrp is highly expressed in a subgroup of hematopoietic stem cells, the so-called "side population".[115] These cells have a high level of plasticity and it may well be that these cells migrate into the liver during extensive damage (see below). The results of Zhou *et al.*[115] suggest that Bcrp has an essential role in maintaining a de-differentiated phenotype, as enforced over-expression delays maturation of the cells. However, its presence in mature, differentiated cells as hepatocytes indicates that Bcrp expression alone is not sufficient for preventing differentiation of cells. Expression of Bcrp can, like Mdr1 and Mrp1, cause multidrug resistance and has been associated with breast cancer.[116]

1.4 Liver regeneration

Under pathophysiological conditions, the expression levels of many hepatic transporters alter markedly. Work described in this thesis focuses on the effect of regeneration of the liver on expression levels of hepatic transporters.

In the normal healthy liver, hepatocytes only rarely proliferate. However, these cells start to divide when the functional capacity of the liver becomes too small. Partial hepatectomy and toxic injury are important stimuli for liver regeneration. In animal models, liver regeneration is often studied after surgical removal of the median and left lateral lobe of the liver. This accounts for approximately 70% of total liver mass. An important advantage of this partial hepatectomy (PHx) model is that there is no inflammation, cell death, or fibrosis.[117]

After PHx, not only the hepatocytes, but also the non-parenchymal cells and the extracellular matrix re-organize. The hepatocytes are the first cells to regenerate, reaching a peak in DNA synthesis at 22-24 hours after PHx. Replication of hepatocytes starts in the periportal areas of the remaining liver. Replication of non-parenchymal cells occurs 24-36 hours later. After a 70% PHx in rats, the liver will grow to its original mass within two weeks after which proliferation stops.[1]

In regenerating liver, one would expect a decreased hepatic functioning during cell division because of partial loss of cell polarity. The expression of most liver-specific proteins is, however, maintained[117] and bile flow and bile salt secretion, when expressed per gram liver, are actually increased.[118,119]

The cytokines TNF-α and IL-6 have essential roles in initiating cell division after PHx, as has been demonstrated using TNF-receptor 1-deficient and IL-6-deficient mice.[120–122] The transcription factors nuclear factor kappa B (NF-κB), activated protein-1 (AP-1) and signal transduction and activators of transcription (STAT) family member 3 (STAT3) are activated by these cytokines and have a key role in transcriptional regulation of gene expression after PHx.

Signaling by TNF-α

TNF-α interaction with the TNF-receptor 1 (TNFR1) results in the association of the receptor's death domains. Subsequently the adaptor protein TNF-receptor-associated death domain (TRADD) binds to the clustered receptor death domains, resulting in the activation of multiple signaling cascades.

Binding of Fas-associated death domain (FADD) to TRADD mediates the activation of caspase 8 and 10. This results in the activation of effector caspases and in apoptosis when anti-apoptotic mechanisms are not effective.[123,124]

Activation of nuclear factor kappa B (NF-κB) (p50-p65 complex) by TNF-α starts with TRADD, and TNF-receptor associated factor-2 (TRAF2)/receptor interacting protein (RIP). This is followed by the activation of NF-κB-inducing kinase (NIK) and inhibitor of κB (IκB) kinase complex (IKK). Alternatively, IKK can be directly activated by reactive oxygen species,[125,126] that are induced by TNF-α.[127,128] NF-κB is retained in the cytoplasm by IκB. For activation of NF-κB, phosphorylation of IκB by IKK is essential, followed by its ubiquitination and degradation by the 26S proteasome. This releases the NF-κB subunits, which then translocate to the nucleus to initiate gene transcription (reviewed by Bradham *et al.*[129] and Wallach *et al.*[130]).

The activation of AP-1 involves binding of TRAF2 to TRADD, followed by the activation of the mitogen activated protein kinase (MAPK) pathways. Through a number of phosphorylation steps, this results in the activation of c-Jun NH_2-kinase (JNK) and p38. These factors can translocate to the nucleus where they phosphorylate transciption factors, ultimately resulting in the activation of transcription factors as c-Jun and c-Fos, members of the AP-1 family (reviewed by Hagemann and Blank[131]). A simplified schematic overview of the TNF-α signaling pathways is shown in Figure 1.4.

Signaling by IL-6

The activation of NF-κB by TNF-α induces IL-6 expression in Kupffer cells. Binding of Il-6 to its receptor results in the activation of intracellular receptor-associated tyrosine kinases (janus kinases, JAKS) and eventually phosphorylation, primarily of STAT3. This phosphorylation induces dimerization of STAT3 and its translocation to the nucleus. Transcriptional activity of STAT3 is further regulated by phosphorylation of specific serine residues. IL-6 can also activate the MAPK pathways.

Progression through the cell cycle subsequently requires the action of growth factors as hepatic growth factor (HGF) and transforming growth factor-alpha. Excellent reviews on this subject have been written by Michalopolous and DeFrances,[117] Taub *et al.*,[132] and Fausto.[133]

1.5 Liver regeneration involving hepatic progenitor cells

After PHx, regeneration of the liver is accomplished by proliferation of hepatocytes and cholangiocytes. However, liver injury after exposure to hepatotoxic chemicals or viral in-

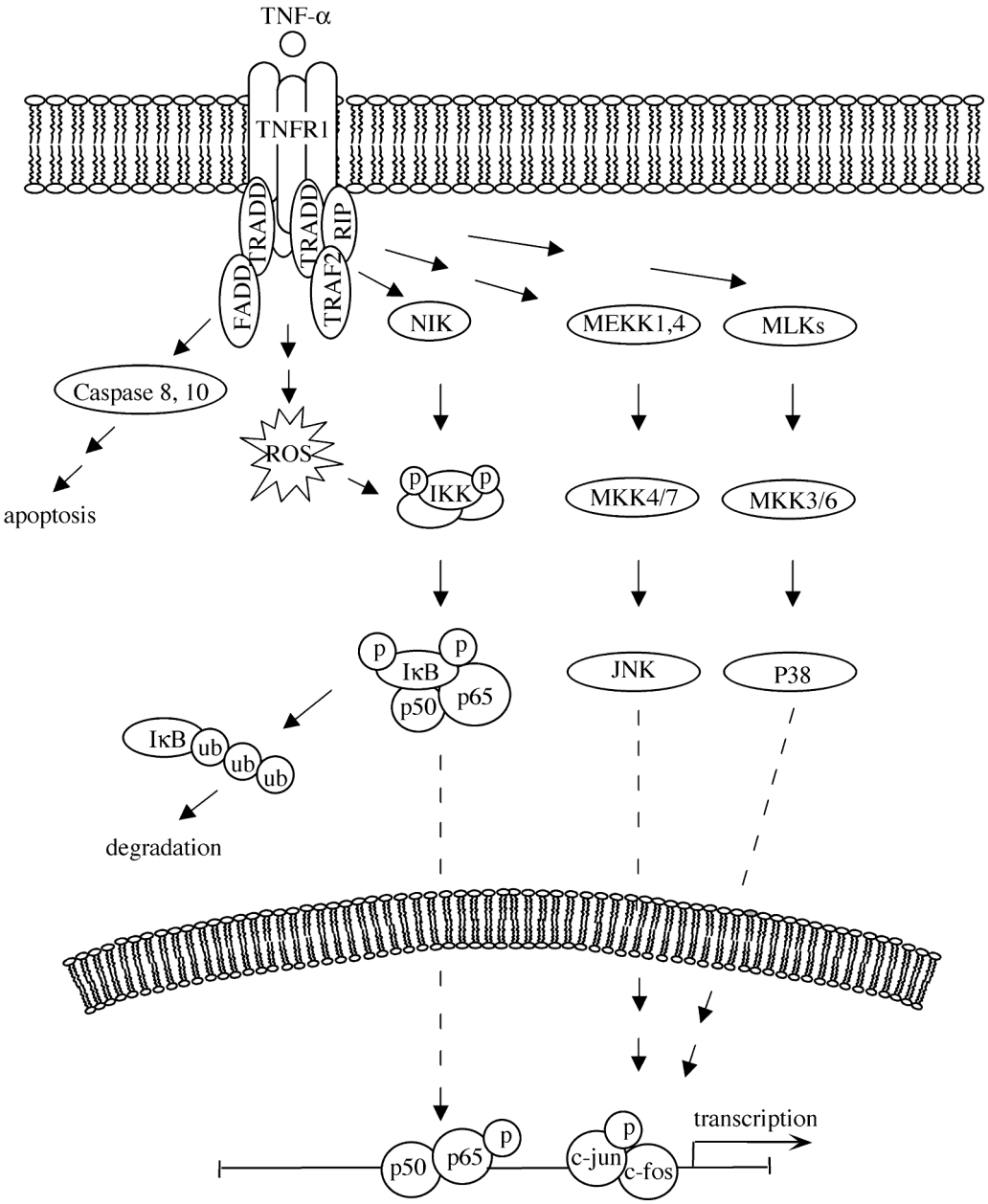

Figure 1.4: Schematic overview of signal transduction pathways activated by TNF-α. Activation of the TNF-receptor (TNFR1) induces the binding of TNF-receptor-associated death domain (TRADD). Via Fas-associated death domain (FADD), caspases are activated. Activation of nuclear factor kappa B (NF-κB) involves the activation of NF-κB-inducing kinase (NIK) via TNF-receptor associated factor-2/receptor interacting protein (RIP). NIK phoshorylates the inhibitor of κB (IκB) kinase complex (IKK). IKK can also be activated by reactive oxygen species (ROS). IKK induces the phosporylation of IκB, resulting in its ubiquitination (ub) and degradation. This releases the NF-κB subunits p50/p65, which translocate to the nucleus and induce gene transcription. Depicted on the right is a simplified overview of the factors involved in the activation of the mitogen activated kinases c-Jun NH_2-terminal kinase (JNK) and p38. Activation involves a member of the MAPK/ERK kinase kinase family (MEKK) or mixed lineage kinase family (MLK) followed by members of the MAPK kinase family (MKK). Translocation of JNK and p38 to the nucleus ultimately results in the activation of transcription factors as c-Jun and c-Fos.

fections is frequently associated with inhibition of the regenerative and functional capacity of hepatocytes and bile duct epithelial cells. Under these conditions, the hepatic progenitor cell compartment becomes activated. There are a number of animal models in which hepatic progenitor cells are specifically activated by induction of severe hepatic injury while the proliferation of hepatocytes is inhibited. One of these models combines treatment of rats with 2-acetylaminofluorene (2-AAF) and PHx. Treatment with 2-AAF decreases the expression of Cyclin E in hepatocytes after PHx. At the same time, expression of p53 and subsequently of p21 are increased. This results in a blockade in the cell cycle.[134]

The localization of the hepatic progenitor cells has been subject of debate for many years. The observations that after pericentral damage liver regeneration was faster than after periportal damage[135] and that damage of the bile ducts by methylene dianiline inhibited the activation of the progenitor cells[136] suggested that the progenitor cells might be localized in or near the biliary tree. Recently, Theise *et al.*[4] evaluated the three-dimensional structure of the ductular reactions by using immunohistochemistry in serial slices of normal liver and of liver with massive necrosis. These authors established that the hepatic progenitor cells are located in or near the canals of Hering. Activation of these progenitor cells results in the generation of oval cells. These cells are small, contain little cytoplasm and have a large ovoid nucleus. Oval cells are cells with a high potential to proliferate: they can be found migrating from the canals of Hering into the periportal region.[4] By using electron microscopy and immunohistochemistry it has been observed that oval cells can have different shapes. Some form irregular duct-like structures,[4] while others are morphologically intermediate between oval cells and hepatocytes.[137] Because of this heterogeneity the total of oval cells is often described as the "oval cell compartment".[138]

The differentiation of oval cells after 2-AAF/PHx in rats has been studied by autoradiography. [^{3}H]-Thymidine initially labeled oval cells, but the label appeared in newly formed hepatocytes 9-11 days after PHx. At this point the expression of α-fetoprotein and γ-glutamyltranspeptidase decreased, and the cells switched from a bile duct cell-like to a hepatocyte-like pattern of cytokeratine expression. Furthermore, cytochrome P450 expression, absent in the oval cells, could be observed in the newly formed hepatocytes.[139,140] In addition to hepatocytes, oval cells can differentiate into bile duct epithelial cells, as demonstrated by immunohistochemistry.[141] These observations have been confirmed by *in vitro* studies. Oval cells in culture can be induced with DMSO or sodium butyrate to differentiate into cells having either hepatocyte-like or bile duct cell-like phenotypes.[142] From these studies it has been concluded that oval cells are bi-potential and capable of differentiation into either hepatocytes or cholangiocytes.

Oval cells are characterized by the expression of specific proteins. They express α-fetoprotein, γ-glutamyltranspeptidase and cytokeratin-19, and can be detected by antibodies such as OV-6, recognizing a common epitope in cytokeratins 14 and 19.[143–145] Furthermore, they express Thy-1, a protein also expressed in hematopoietic stem cells.[146] These markers help in the identification of oval cells and allow the isolation and *in vitro* characterization of highly enriched oval cell populations.

In humans, many types of liver injury induce an increase in bile duct-like structures near the portal tract, the so-called "ductular reaction". Cells in these structures can dif-

ferentiate towards cholangiocytes or hepatocytes. Differentiation towards biliary epithelial cells is seen in extrahepatic biliary obstruction. This is comparable to the bile duct ligation model in rats. Proliferation of cells that resemble both cholangiocytes and hepatocytes is observed in primary biliary cirrhosis, chronic active hepatitis, and submassive necrosis. Differentiation towards hepatocytes is most pronounced during regeneration after submassive necrosis.[147–149]

1.6 Bone marrow-derived stem cells

In addition to mature hepatocytes and hepatic progenitor cells, it has recently been shown that circulating bone marrow stem cells can differentiate into functional liver cells. This was first demonstrated in rats that had undergone bone marrow transplantation prior to severe hepatic damage. In the livers of these animals, mature hepatocytes and cholangiocytes could be detected that were derived from the donor and thus had to originate from bone marrow-derived cells.[150] Similar observations were subsequently made in humans. In liver samples from female recipients of male bone marrow or from male recipients of a female liver, hepatocytes containing the Y-chromosome were detected. The number of bone marrow-derived cells in these livers was however highly variable.[151,152]

The ABC transporter gene expression of bone marrow-derived stem cells is largely unknown. Although the expression of ABC transporters has been studied in different bone marrow cell types, knowledge regarding their expression in purified stem cell populations is only limited. Only recently, Zhou *et al.*[115] reported that murine hematopoietic stem cells with the side-population phenotype ($ckit^+Sca^+Lin^-$) express *Bcrp*, *Mdr1a*, *Mrp1*, *Mrp3*, and *Mrp4* mRNA.

From these observations it can be concluded that bone marrow-derived stem cells may play a role in hepatic regeneration. However, as only limited numbers of these cells are present in diseased liver, it seems unlikely that these cells have the capacity to fully regenerate a damaged liver. Further characterization of these cells may lead to novel methods to stimulate the repopulation process, which may result in novel therapies for chronic liver disease in the future.

1.7 Outline and aim of this thesis

One may assume that cells that proliferate during severe liver damage require well-developed defense mechanisms to withstand cellular damage under conditions of severe metabolic stress. One potential mechanism of cellular protection is the expression of efflux pumps, belonging to the ABC superfamily of membrane transporters, which are able to dispose toxic metabolites from cells. The aim of the work described in this thesis was to characterize the expression of ABC transporter genes in different hepatic cell types after experimental or disease-related liver damage in animal and humans.

In work described in **chapter 2** the expression of hepatic transport systems involved

in bile formation was studied during liver regeneration that occurs after partial hepatectomy (PHx) in rats. mRNA and protein levels of transporters were analyzed in detail in hepatocytes and related to the actual bile flow rate and the secretion rates of bile salts, bilirubin conjugates, and GSH under these circumstances.

Subsequently, in **chapter 3**, ABC transporter gene expression was studied in a rat model for hepatic progenitor cell activation. The progenitor cell compartment was induced by treating rats with 2-acetylaminofluorene (2-AAF) followed by PHx. Gene expression was studied in total liver, both on mRNA and on protein level. In addition, different cell fractions were isolated. This allowed for a comparison of ABC transporter gene expression in hepatic progenitor cells, hepatocytes, and cholangiocytes.

As progenitor cells are also activated during severe liver diseases in humans, ABC transporter gene expression was studied by immunohistochemistry and real-time detection PCR in human liver specimens obtained from patients diagnosed with primary biliary cirrhosis, chronic hepatitis C, or submassive liver cell necrosis and compared with normal human liver. The results are described in **chapter 4**.

We observed in chapter 2 that the expression of the ABC transporter gene *Mdr1b* was highly increased in hepatocytes after partial hepatectomy. To get insight in the molecular mechanism involved in the transcriptional control of *Mdr1b* during liver regeneration, the mechanism of up-regulation of *Mdr1b* in hepatocytes by TNF-α was elucidated (**chapter 5**). TNF-α can signal through various pathways, including NF-κB and p53, transcription factors for which binding sites in the *Mdr1b* promoter have been identified. By using specific inhibitors, we could determine which signal transduction pathway was involved in TNF-α-induced *Mdr1b* expression. Finally, the transcription site that is involved in the up-regulation was determined using transient transfection assays.

This thesis is conluded with a summary of the obtained data.

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Chapter 2

Regulation of hepatic transport systems involved in bile secretion during liver regeneration in rats

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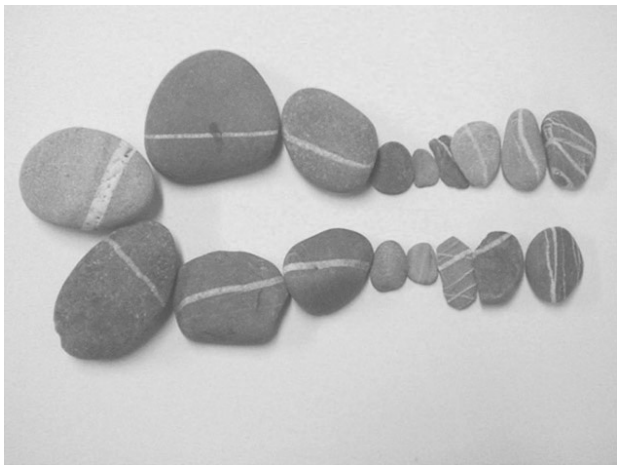

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2.1 Abstract

We investigated the expression of hepatic transport systems involved in bile secretion during liver regeneration after partial hepatectomy (PHx) in rats. Initial studies showed maximal BrdU incorporation 24 hours after PHx. Therefore, transporter expression and bile secretion were analyzed in detail at this time. The mRNA levels of the multidrug resistance genes *Mdr1a* and *Mrp1* slightly increased, whereas *Mdr1b* mRNA levels showed an extensive increase after PHx. The mRNA levels of the conjugate transporter *Mrp2* decreased slightly, whereas Mrp2 protein levels did not change. Bilirubin secretion did not change, but the biliary glutathione secretion markedly decreased and the hepatic GSH content increased. The mRNA levels of the bile salt uptake transporters *Ntcp*, *Oatp1*, and *Oatp2* and the bile salt exporter *Bsep* all decreased with *Ntcp* showing the most prominent decrease. Protein levels of Ntcp dramatically decreased whereas Oatp2 only slightly decreased. Oatp1 protein expression slightly increased and Bsep protein levels did not change. Decreased levels of bile salt uptake systems were associated with a 10-fold increase in the plasma bile salt concentration, yet, bile flow and bile salt secretion were increased when expressed per gram liver and unaffected when expressed on the basis of body weight. In conclusion, during the initial phase of rat liver regeneration Ntcp is down-regulated whereas other transporter proteins involved in bile secretion are only slightly affected. Despite increased serum bile salt levels the remnant liver is not cholestatic: bile flow is maintained by uptake of bile salts probably via Oatp isoforms and their secretion via Bsep.

2.2 Introduction

Under normal circumstances hepatocytes are quiescent cells that fulfill a variety of tissue-specific functions such as xenobiotic biotransformation and bile formation. The latter is crucial for maintenance of cholesterol homeostasis, absorption of dietary fats, and removal of waste products and their metabolites. Bile formation is an osmotic process that critically depends on active secretion of osmotically active compounds from liver into the bile canalicular lumen. Bile salts and glutathione (GSH) are considered the major contributors to bile flow generation in rodents.[1,2] After removal of part of the liver, the remaining hepatocytes undergo a synchronized process of DNA synthesis and cell division. DNA synthesis starts in periportal hepatocytes with a first peak 22-24 hours after PHx. DNA replication of non-parenchymal cells follows 24-36 hours later. Within a few weeks the liver has grown to its original mass and the hepatocytes become quiescent again.[3,4]

Although one would expect a decreased hepatic excretory and secretory function during cell division because of partial loss of cell polarity, earlier investigations have actually shown increased bile flow and increased secretion of bile salts, when expressed per gram remaining tissue, during liver regeneration in rodents.[5–8] The relationship between bile formation and the expression of hepatic transport systems involved herein have not been defined under these conditions. The uptake of cholephilic compounds from sinusoidal blood into hepatocytes is largely dependent on the Na^+/taurocholate cotransporting polypeptide Ntcp[9] and the Na^+-independent organic anion transporting polypeptides Oatp1[10] and Oatp2.[11] Ntcp transports conjugated bile salts (*e.g.*, taurocholate) and, to a lesser degree, unconjugated bile salts (*e.g.*, cholate). Both Oatp1 and Oatp2 can transport a wide variety of structurally unrelated compounds including bromosulfophthalein, taurocholate, cholate, leukotriene C_4, *S*-dinitrophenyl glutathione, and steroid conjugates (*e.g.*, estradiol-17β-glucuronide).[12,13] Recently, it has been demonstrated that hepatic uptake of organic anions by Oatp1 can be driven by the exchange with intracellular GSH.[13]

Secretion of cholephilic compounds from hepatocytes into bile is largely dependent on members of the ATP-binding cassette protein superfamily[1,2]: the P-glycoprotein (Pgp) subfamily and the multidrug resistance protein (MRP) subfamily. Until now four members of the Pgp subfamily have been cloned. Mdr1a and Mdr1b are present at low levels at the canalicular membrane of normal rodent liver. Overexpression of Mdr1a/Mdr1b confers multidrug resistance against a broad variety of natural toxins and drugs. In contrast to Mdr1, the canalicular expression of Mdr2 is high. This transporter functions as a flippase that translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane for secretion into bile. Finally, the sister of Pgp (Spgp) has recently been identified as the major canalicular bile salt export pump (Bsep).[14]

The expression of four members of the MRP subfamily has been demonstrated in rat liver so far. In normal liver, Mrp1 is present at low levels. Mrp1 is able to transport mostly multivalent anionic conjugates such as bilirubin diglucuronide and GSH S-conjugates, including the lipid peroxidation product leukotriene C_4. In contrast to Mrp1, its homologue Mrp2 is highly expressed in the liver and is located at the canalicular membrane. Mrp2 has the same substrate specificity as Mrp1. Two other Mrp homologues are also present

in the liver, Mrp3[15,16] at low levels and Mrp6[16,17] at relatively high levels. Mrp6 is located at the lateral plasma membrane of hepatocytes and transports the anionic cyclopentapeptide endothelin receptor antagonist BQ123.[17] The endogenous substrate of Mrp6 is not yet known.

Earlier studies have shown a strongly decreased expression of Ntcp[18] and a clearly increased expression of Mdr1,[19–21] especially Mdr1b,[22] after PHx in rats. However, in these studies neither the flow of bile nor the secretion of bile constituents and the expression of other transporters located at the basolateral and canalicular membrane were determined. For normal production of bile, the organic anion transporter Mrp2 and the bile salt transporter Bsep are essential. Therefore, we examined the expression of these transporters and related this to bile flow and secretion of bile salts, bilirubin conjugates, and GSH. Under conditions where basolateral transporters are down-regulated, sinusoidal uptake rather than canalicular secretion may become rate-limiting for overall bile formation. Therefore, we also investigated the expression of the basolateral uptake transporters Ntcp, Oatp1, and Oatp2 during liver regeneration.

2.3 Materials and methods

Animals

Pathogen-free male Wistar rats (220-260 g) were purchased from Harlan-CPB, Zeist, the Netherlands. They were kept under routine laboratory conditions at the Central Animal Laboratory of the University of Groningen. The rats received standard laboratory chow and had free access to food and water. This study was approved by the Local Committee for Care and Use of Laboratory Animals.

Experimental design

PHx was performed according to the technique of Higgins and Anderson.[23] Sham-operated animals received the same treatment as partial hepatectomized rats, including manipulation of the liver, but without hepatectomy. For measurement of transporter mRNA levels at different time points after PHx (sham, 3, 12, 24, 48, and 216 hours, n=4), livers were perfused with phosphate-buffered saline (PBS) under sodium pentobarbital anesthesia (60 mg/kg, intraperitoneally), removed, cut into small pieces, snap-frozen in liquid nitrogen and stored at -80°C until further use. In another experiment, rats underwent PHx (n=3) or sham operation (n=3) and rat hepatocytes were isolated 24 hours later by two-step collagenase perfusion as described previously.[24] The hepatocytes were used immediately for isolation of mRNA and plasma membranes. Bile was collected 24 hours after PHx (n=5) or after sham operation (n=5) by cannulation of the common bile duct under sodium pentobarbital anesthesia (60 mg/kg, intraperitoneally). After a calibration time of 5 minutes, bile was collected for 5 minutes and immediately frozen in liquid nitrogen. For determination of bile GSH levels, a second fraction of bile was collected for 15 minutes in a pre-weighted tube with 50 μL 10% 5-sulfosalicylic acid (Sigma, St. Louis, MO), weighted again, centrifuged for 5 minutes at 13,000 rpm and frozen in liquid nitrogen.

Blood was obtained by cardiac puncture, mixed with EDTA and centrifuged. Samples of 600 μL plasma were frozen in liquid nitrogen. For GSH determination, 800 μL plasma was immediately mixed with 200 μL ice cold 10% 5-sulfosalicyclic acid and centrifuged for 5 minutes at 13,000 rpm. Supernatant was frozen in liquid nitrogen.

Analytical methods

Bile flow was determined gravimetrically, assuming a density of 1.0 mg/μL. Total bile salts, phospholipids, and cholesterol in plasma and bile were measured as described.[25] Total GSH levels (GSH+GSSG) in plasma, liver, and bile were determined according to Griffith.[26] Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and total bilirubin were determined by routine clinical chemistry.

RNA isolation and reverse transcription polymerase chain reaction

Because the expression of the *Mdr1a*, *Mdr1b*, and *Mrp1* genes is very low in normal rat liver, we performed semiquantitative reverse-transcriptase polymerase chain reactions (RT-PCR) to detect these and other transporter gene products. Total RNA was isolated from frozen rat liver and isolated hepatocytes using TRIzol Reagent (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Subsequently, mRNA was isolated using the Oligotex mRNA mini-kit (Qiagen GmbH, Hilden, Germany). Single stranded cDNA was synthesized from 2.5 μg mRNA using 0.5 nmol random primers (Pharmacia, Uppsala, Sweden) and 50 U AMV Reverse-Transcriptase (Promega, Madison, WI) in a buffer containing 50 mmol/L Tris-HCl, 50 mmol/L KCl, 10 mmol/L DTT, 10 mmol/L $MgCl_2$, 0.5 mmol/L spermidine (Promega), 50 U RNA guard (Pharmacia), and 1.25 mmol/L of each dNTP (Pharmacia) in a total volume of 100 μL. RT was performed for 10 minutes at 25°C and for 1 hour at 50°C and the samples were subsequently heated for 5 minutes at 95°C to terminate the RT reaction. With the complementary DNA (cDNA) obtained, a PCR reaction was performed using 3 μL of the RT reaction mixture supplemented with 2.5 U Taq polymerase (Pharmacia), 50 pmol sense and 50 pmol antisense primer. The final reaction volume was 50 μL. The tubes were incubated in a Gene Amp PCR system 2400 (Perkin-Elmer, Norwalk, CT) at 95°C for 5 minutes to denature the primers and cDNA. The cycling program was 95°C for 30 seconds, 54°C to 60°C for 30 seconds, 72°C for 30 seconds and for 5 minutes in the last cycle and comprised 20 to 33 cycles. For each primer set, an increasing number of PCR-cycles with otherwise fixed conditions was performed to determine the optimal number of cycles to be used. This was determined to be halfway the exponential phase. *β-Actin* was used as internal control. Primers specific for *Ntcp*[9] (sense: 5'-ATG CCC TTC TCT GGC TTT CT-3'; antisense: 5'-GCT CCA TGG TTC TGA TGG TT-3'), *Oatp1*[10] (sense: 5'-AAG CGA AGA AGC TGG AAA CA-3'; antisense: 5'-CAC CAC AGG TCT GTG CAG TT-3'), *Oatp2*[11] (sense: 5'-TGC ACA CTT AGC ATT CTG GC-3'; antisense: 5'-TGC ATG TAA CCC AAC TCC AA-3'), and *β-Actin*[27] (sense: 5'-CCT AAG GCC AAC CGT GAA AAG-3'; antisense: 5'-TCT TCA TGG TGC TAG GAG CCA-3') were used resulting in amplified products of 500, 302, 496, and 646 bp, respectively. All other PCR-primers used in this study were described previously.[28] All PCR-products were sequenced in order to confirm specificity of

the PCR-primers. In each experiment, water was used as a negative control. Ten microliters of each PCR product were loaded on a 2.5% agarose gel and stained with ethidium bromide.

Isolation of crude plasma membranes from isolated hepatocytes

Freshly isolated hepatocytes were washed in Krebs buffer (118 mmol/L NaCl, 5 mmol/L KCl, 1.1 mmol/L $MgSO_4$, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 10 mmol/L D(+)glucose, 10 mmol/L Hepes, 1% bovine serum albumin [pH 7.42]) and pelleted at $500g$ for 8 minutes. For permeabilization, cells were stirred in 35 mL 1 mmol/L $NaHCO_3$/0.1 mmol/L PMSF for one hour at 4°C. After centrifugation at 90,000g for 30 minutes at 4°C, the pellet was resuspended in 15 mL 250 mmol/L sucrose/0.1 mmol/L PMSF and homogenized 50 times with a tight Dounce homogenizer. A centrifuge tube was loaded with 15 mL 38% (wt/wt) sucrose and 15 mL hepatocyte homogenate, respectively. After centrifugation at 90,000g for 90 minutes at 4°C, the 38% fraction was washed in 1 mmol/L $NaHCO_3$/0.1 mmol/L PMSF and centrifuged at 47,000g for 30 minutes at 4°C. The pellet was resuspended in 1 mmol/L $NaHCO_3$/Complete protease inhibitor cocktail (1 tablet/50 mL, Boehringer Mannheim GmbH, Mannheim, Germany) by 15 times in-and-out suctioning through a 25-gauge needle and stored at -80°C until further use.

Antibodies

Antibodies against Mrp2 and Bsep were described before.[28] Antibodies against Ntcp,[29] Oatp1,[30] and Oatp2[31] were kind gifts from Dr. Bruno Stieger and Dr. Peter J. Meier (Zürich, Switzerland). Mouse mAb C219 (Signet Laboratories Inc, Dedham, MA) was used to detect all pgps. Mouse mAb against dipeptidyl peptidase IV (DppIV/CD26) was purchased from Endogen (Woburn, MA). Na^+K^+-ATPase antibodies raised in goats against the α- and β-subunits of Na^+K^+-ATPase were kindly provided by Dr. Wilbert Peters, Nijmegen, the Netherlands.

Western blot analysis

The protein concentrations in membrane fractions were determined with the DC Protein Assay from Bio-Rad (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. Fifty micrograms of membrane proteins was fractionated on a 7.5% (Mrp2, Bsep, C219, and DppIV) or 10% (Ntcp, Oatp1, Oatp2, and Na^+K^+-ATPase) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose (Amersham International plc, Buckinghamshire, UK), using a tankblotting system according to the manufacturer's instructions (Bio-Rad Laboratories). For DppIV detection, samples were boiled in sample buffer for 5 minutes before loading on SDS-PAGE. BDH molecular-weight standards (42,700-200,000 molecular-weight; BDH Ltd., Dorset, UK) were used as marker proteins. The blots were stained with Ponceau S-solution (0.1% Ponceau S [wt/vol] in 5% acetic acid [vol/vol]; Sigma) to confirm similar protein concentrations in every lane. The blots were incubated with the first antibody diluted in PBS containing 4% SKIM milk (Fluka BioChemica, Buchs, Switzerland) and 0.1% polyoxyethylene sorbitan monolaurate (Tween-20, Sigma), washed in PBS/0.1% Tween-20, subsequently

incubated with horseradish peroxidase-labeled swine anti-rabbit IgG or rabbit anti-mouse IgG diluted in PBS/4% SKIM milk/0.1% Tween-20 (dilution 1:2000; DAKO, Glostrup, Denmark) and finally developed using Pierce SuperSignal Chemiluminescent Substrate Luminol/Enhancer (Pierce, Rockford, IL).

Statistical analysis

The data resulting from the two experimental groups were expressed as the mean ± SEM. An unpaired Students t test was used to compare the means between the two groups. A P value < 0.05 was considered significant.

2.4 Results

Time-dependent changes in transporter mRNA levels after PHx

In an initial experiment we characterized the expression of several hepatic transport systems at 3, 12, 24, 48, and 216 hours after PHx using semiquantitative RT-PCR (Figure 2.1, n=4). In the Pgp subfamily, mRNA levels of both *Mdr1a* and *Mdr2* slightly increased after PHx. The increased expression of *Mdr1a* was maximal 24 hours after PHx, whereas *Mdr2* expression was maximal 48 hours after PHx. *Mdr1b* expression increased after PHx, with a maximum at 24 hours, whereas *Bsep* mRNA levels showed a slight decrease (Figure 2.1). In the MRP subfamily, *Mrp1* mRNA levels were increased 3 hours after PHx and remained increased until at least 48 hours. *Mrp2* mRNA levels were dramatically decreased 12 hours after PHx, but returned to almost normal levels at later time points. The expression of the gene encoding γ-glutamylcysteine synthetase (*γ-Gcs*), the key enzyme in glutathione synthesis, was maximally elevated 24 hours after PHx. The mRNA levels of the basolateral uptake transporters *Ntcp*, *Oatp1*, and *Oatp2* were reduced 12 hours after PHx and recovered thereafter.

After this initial study, we chose 24 hours after PHx to investigate in more detail the bile flow and secretion of bile salts, cholesterol, phospholipids, GSH, and conjugated bilirubin after PHx. This time point was chosen because 24 hours after PHx a peak in DNA synthesis was found, as measured by BrdU incorporation (data not shown).

Characteristics of partial hepatectomized rats

The body weight of sham-operated rats was 243 ± 11.7 g before and 244 ± 8.7 g 24 hours after surgery. The body weight of PHx rats was 241 ± 9.2 g before and 227 ± 9.5 g 24 hours after surgery. On average, 7.7 ± 0.7 g of liver were removed. Liver weights from sham-operated animals were used to calculate the % liver weight/body weight (4.8%). From this, the percentage of liver removed from the partial hepatectomized group was estimated to be 66%.

Plasma parameters in partial hepatectomized rats

Compared with sham-operation, PHx led to significantly increased AST, ALT, and ALP plasma levels 24 hours after operation (AST: 93 ± 12 vs. 383 ± 47 U/L, ALT: 52 ± 8 vs. 203 ± 32 U/L, and ALP: 139 ± 13 vs. 256 ± 26 U/L, all $P<.05$). The concentration of

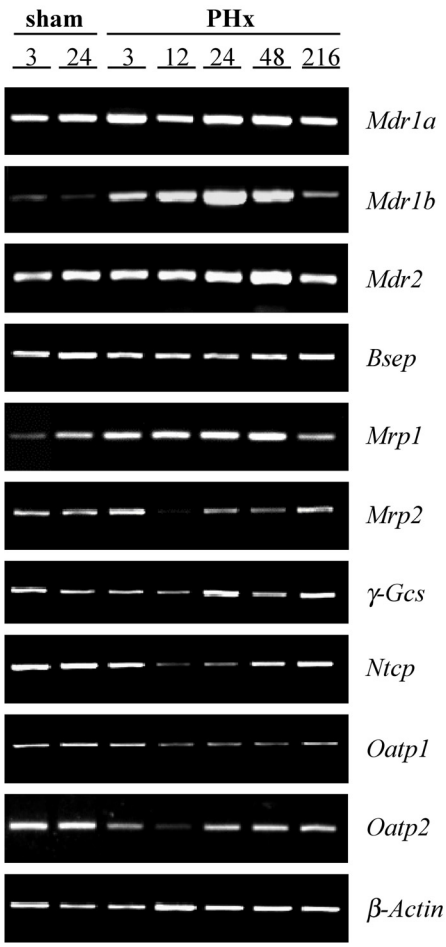

Figure 2.1: Time-dependent expression of transporter mRNA in total rat liver after partial hepatectomy. Total rat liver RNA was isolated and RT-PCR was performed as described in Materials and methods. RT-PCR-products are indicated on the *right side* of each gel and time points (in hours) after partial hepatectomy *above* the figure. Data are representative for n=4 per time point.

bile salts in plasma increased 10-fold after PHx (Table 2.1). Also bilirubin levels in plasma increased significantly, although less pronounced (1.6-fold, Table 2.1). Plasma cholesterol levels decreased to 76% of control levels ($P<.05$) and plasma phospholipids to 89% (not significant). Total GSH in plasma did not change after PHx (Table 2.1).

Bile secretion in partial hepatectomized rats

In bile neither the bile salt nor the bilirubin concentration changed significantly after PHx (Table 2.2). Cholesterol and phospholipid concentrations both decreased significantly to 55% and 54% of control values. The biliary GSH concentration decreased to 23% (Table 2.2), whereas GSH levels in the liver increased significantly from 6.6 ± 1.2 to 11.5 ± 1.6 μmol/g liver. When expressed per gram liver, bile flow was increased 1.75-fold after PHx (Table 2.3). The secretion of bile salts and bilirubin was also increased (1.9 [$P<$

Table 2.1: Plasma composition in sham-operated and partial hepatectomized rats

	Sham	24 hr PHx
Bile salts[1]	36.3 ± 2.7	380.3 ± 99.0*
Total bilirubin[1]	3.60 ± 0.12	5.73 ± 0.55*
Cholesterol[2]	1.05 ± 0.06	0.80 ± 0.06*
Phospholipids[2]	2.18 ± 0.08	1.95 ± 0.08
Total GSH[1]	12.8 ± 1.0	13.1 ± 2.1

Data represent the means ± SEM of n = 6 rats per group, [1]μmol/L, [2]mmol/L, $^\star P<0.05$

.05] and 1.5-fold [ns], respectively). Cholesterol and phospholipid secretion did not change significantly but the secretion of GSH decreased to 39% of control levels. When calculated per 100 gram body weight (Table 2.3), bile flow and the secretion of bile salts and bilirubin did not change significantly, whereas the secretions of cholesterol, phospholipids, and GSH were decreased.

Transporter mRNA levels in hepatocytes isolated from remnant livers 24 hours after PHx

To be certain that the observed changes in transporter mRNA levels as shown in Figure 2.1 are taking place in hepatocytes rather than in other liver cell types, RT-PCR was performed on RNA obtained from hepatocytes that were isolated 24 hours after PHx (Figure 2.2). Twenty-four hours after PHx, a very strong increased expression of *Mdr1b* mRNA was seen, whereas *Mdr1a* and *Mdr2* mRNA levels only slightly increased. The mRNA levels of the bile salt transporter *Bsep* and the major conjugate transporter *Mrp2* slightly decreased. The mRNA levels of *Mrp1* and *γ-Gcs* were increased. Twenty-four hours after PHx, *Ntcp* mRNA was dramatically decreased. *Oatp1* and *Oatp2* mRNA levels were also decreased, but less pronounced then that of *Ntcp*. *β-Actin* mRNA was slightly increased 24 hours after PHx, as reported before.[21,32–34] This was confirmed by Northern blot with 28S as control for equal RNA loading (data not shown).

Transporter protein levels in hepatocytes isolated from remnant livers 24 hours after PHx

Hepatocytes isolated 24 hours after PHx were used to obtain crude plasma membrane fractions for Western blot analysis (Figure 2.3). After PHx, no changes of Mrp2 and Bsep protein levels were observed. The C219 signal, detecting all Pgps, was dramatically

Table 2.2: Bile composition in sham-operated and partial hepatectomized rats

	Sham	24 hr PHx
Bile salts[1]	54.5 ± 3.5	59.4 ± 1.9
Total bilirubin[2]	41.6 ± 1.4	34.9 ± 3.5
Cholesterol[1]	0.73 ± 0.06	0.40 ± 0.03*
Phospholipids[1]	6.7 ± 0.4	3.6 ± 0.4*
Total GSH[1]	3.6 ± 0.8	0.83 ± 0.23*

Data represent the means ± SEM of n = 5 rats per group, [1]μmol/L, [2]mmol/L, $^\star P<0.05$

Table 2.3: Bile flow and biliary secretion rates in sham-operated and partial hepatectomized rats

	Sham	24 hr PHx
Secretion per gram liver		
Bile flow[1]	1.5 ± 0.1	2.6 ± 0.4*
Bile salt[2]	80 ± 4	152 ± 22*
Total bilirubin[2]	0.06 ± 0.001	0.09 ± 0.02
Cholesterol[2]	1.1 ± 0.1	1.0 ± 0.2
Phospholipids[2]	9.9 ± 0.7	9.4 ± 1.6
Total GSH[2]	5.4 ± 1.3	2.1 ± 0.6*
Secretion per 100 gram body weight		
Bile flow[3]	7.1 ± 0.3	6.3 ± 1.2
Bile salt[4]	383 ± 18	370 ± 59
Total bilirubin[4]	0.29 ± 0.01	0.22 ± 0.04
Cholesterol[4]	5.2 ± 0.6	2.5 ± 0.4*
Phospholipids[4]	47.7 ± 3.2	22.6 ± 3.9*
Total GSH[4]	26.4 ± 6.7	5.2 ± 2.3*

Data represent the means ± SEM of n = 6 rats per group, [1]μL/g liver/min, [2]nmol/g liver/min, [3]μL/100 g b.wt./min, [4]nmol/100 g b.wt./min, $^\star P<0.05$

increased after PHx. Protein levels of the uptake transporter Ntcp dramatically decreased, whereas Oatp2 protein levels only slightly decreased (Figure 2.3). The appearance of Ntcp protein bands as doublets has been described previously and is probably due to partial deglycosylation of the protein.[29] Twenty-four hours after PHx, Oatp1 protein levels were slightly increased, despite decreased Oatp1 mRNA levels. As controls for the membrane isolation procedure, DppIV (canalicular protein) and Na^+K^+-ATPase (α- and β-subunits, basolateral protein) were used and no significant changes in their expression were found (Figure 2.3).

2.5 Discussion

In this study we compared the effects of PHx on bile secretion with the expression of a number of transporters involved in bile formation. Expressed per g liver, PHx led to an increase in bile flow, an increased secretion of bile salts, an unchanged secretion of conjugated bilirubin and a decreased secretion of GSH. PHx had no effect on the secretion of cholesterol and phospholipids per g liver. Despite a significantly increased secretion of bile salts and a decreased secretion of GSH, the mRNA and protein levels of Bsep and Mrp2, the transporters thought to be involved in bile salt[14] and GSH secretion,[35] showed no major changes after PHx. The strong up-regulation of *Mdr1b* mRNA and the minor increase in *Mdr1a* and *Mdr2* mRNA are in agreement with earlier studies.[19–22] In this study we show that *Mrp1* mRNA is up-regulated after PHx. This is in agreement with

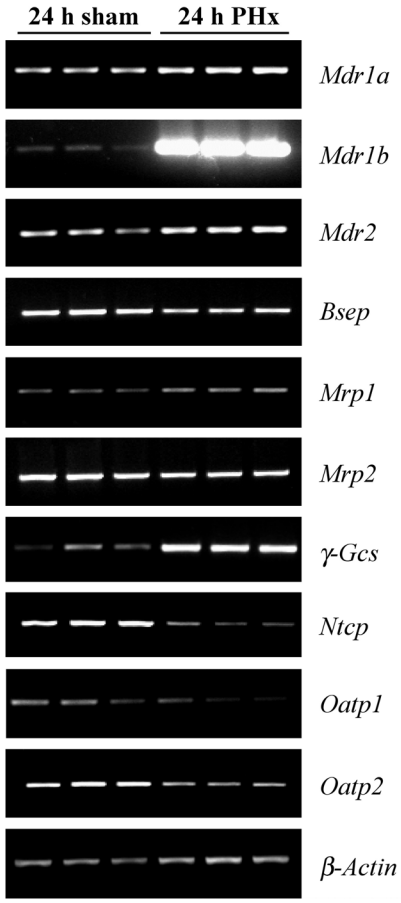

Figure 2.2: Expression of transporter mRNA in isolated rat hepatocytes 24 hours after partial hepatectomy. RNA was isolated from freshly isolated rat hepatocytes and RT-PCR was performed as described in Materials and Methods. RT-PCR-products are indicated on the *right side* of each gel and treatment group *above* the figure. Results from hepatocyte isolations from three sham-operated and three PHx rat livers are shown.

the increased levels of Mrp1 seen in proliferating hepatocyte-derived cells.[36] Up-regulation of *Mrp1* and *Mdr1b*, which are normally expressed at very low levels, may increase the resistance of hepatocytes against products of cellular oxidative stress reactions such as lipid peroxidation products.[28]

As shown in a study from Green *et al.*[18] and confirmed in our study, PHx leads to drastically decreased Ntcp mRNA and protein levels. At least in part, this down-regulation is induced by the increased levels of bile salts after PHx. These activate the bile acid receptor FXR/BAR. Active as a heterodimer with RXR-α, FXR induces the transcription of the small heterodimer partner-1 (Shp1). Shp-1 in turn inhibits RXR:RAR,[37] important for the basal expression levels of Ntcp.[38] With the reduction in Ntcp expression, the Na^+-dependent uptake of bile salts will be seriously decreased. Twenty-four hours after PHx, Oatp1 protein levels were slightly increased, despite decreased mRNA levels. This could be

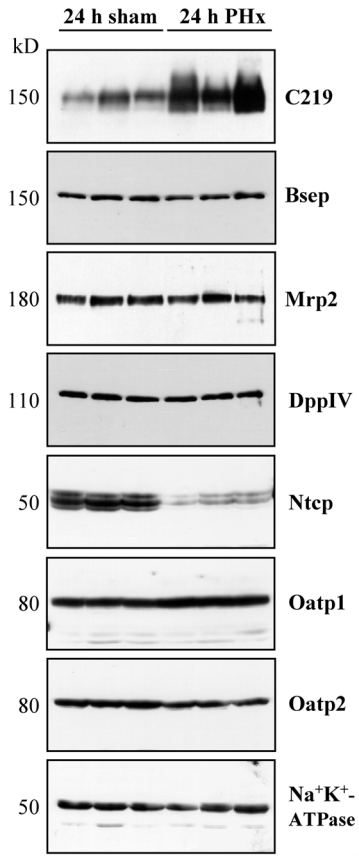

Figure 2.3: Western blots of transporter proteins in crude plasma membranes of isolated rat hepatocytes 24 hours after sham operation or partial hepatectomy. Fifty micrograms of crude plasma membranes was separated on 7.5% (Mrp2, Bsep, C219, and DppIV) or 10% (Ntcp, Oatp1, Oatp2, and Na^+K^+-ATPase) SDS-PAGE gel and transferred to nitrocellulose. The proteins were visualized using the corresponding antibodies, as indicated on the *right side* of each blots. C219 recognizes all Pgps. Apparent molecular weights are indicated on the *left side* of each blot, the treatment group *above* the blot. Results from hepatocyte isolations from three sham-operated and three PHx rat livers are shown.

the result of a decreased breakdown of the Oatp1 protein during liver regeneration. It has to be noted however, that little information is yet available on the mechanism of degradation of the various transporters. Oatp2 protein levels were only slightly decreased 24 hours after PHx. This preservation of Oatp1 and Oatp2 protein expression could compensate, at least in part, for the markedly decreased Ntcp expression (Figure 2.3) and ensure ongoing basolateral uptake of bile salts and other organic anions. However, because of their similar K_M values for taurocholate (Ntcp $\sim$ 25 μM;[39] Oatp1 $\sim$ 50 μM;[40] Oatp2 $\sim$ 35 μM[11]), Oatp1 and Oatp2 would be fully saturated in the absence of Ntcp expression and can obviously not prevent a marked increase in the plasma concentration of bile salts. Nevertheless, we postulate that after PHx bile salts are taken up mainly via the Oatp isoforms. Oatp2 is an especially good candidate because it is expressed at high levels in rat liver. Uptake of

bile salts via Oatp1 and Oatp2 will be facilitated because of the 10-fold increased serum bile salt levels. Moreover, Oatp1-mediated uptake of bile salts is also stimulated by higher intracellular GSH concentrations,[13] as found during liver regeneration by us and others.[41]

Under normal circumstances only periportal hepatocytes are involved in uptake (by Ntcp) and secretion (by Bsep) of bile salts. Because of the decreased Ntcp expression and the high serum bile salt levels, Oatp-expressing midzonal and pericentral hepatocytes are likely to participate in bile salt uptake during liver regeneration. This would explain the increased bile salt secretion capacity per g liver we observed. Furthermore, participation of pericentral hepatocytes in bile salt clearance has been shown under conditions of high serum bile salts.[42–45] Moreover, Baumgartner *et al.*[46] reported a decreased metabolic zonation of bile salt processing after PHx in the isolated perfused rat liver. Such a shift of bile salt processing towards the pericentral hepatocytes in regenerating liver could protect periportal hepatocytes against the high levels of toxic bile salts that have to be handled by a decreased liver mass. Apparently, high plasma bile salt levels are less toxic to the organism than high intracellular bile salt levels, probably because plasma bile salts are bound to plasma components.

This study also shows that the secretion of GSH is significantly decreased after PHx per g liver, whereas the secretion of conjugated bilirubin did not change. Since the GSH concentrations and the mRNA of γ-*Gcs*, the rate-limiting enzyme in GSH synthesis, are increased after PHx, these findings can either be explained by a down-regulation of the implicated transporters or by competitive inhibition of GSH transport by other intracellular solutes. It has been reported that canalicular GSH secretion might be mediated by Mrp2.[35] Mrp2 mRNA levels are decreased at early, but not later time points, whereas no changes in Mrp2 protein levels were observed. Therefore, competition between the low-affinity substrate GSH and some high-affinity substrates such as bilirubin glucuronides[47] is the most likely explanation for the reduced biliary GSH secretion.

In summary, the remnant liver is exposed to increased bile salt concentrations after PHx. Down-regulation of Ntcp impairs the uptake of bile salts by this Na^+-dependent, high-affinity bile salt uptake transporter. This will prevent or reduce the toxic accumulation of bile salts in periportal hepatocytes, which constitutes the main proliferating compartment in the regenerating liver. Oatp isoforms may take over the predominant role of Ntcp in bile salt uptake, thereby recruiting midzonal and pericentral hepatocytes for bile salt uptake and maintaining unchanged bile salt secretion expressed per 100 g body weight or, in other words, the small remnant liver is fully capable to maintain hepatic flux of the circulating bile salt pool. By adapting the expression of uptake systems, the remnant liver can handle the increased bile salt load per cell without overloading periportally located proliferating hepatocytes.

2.6 Acknowledgment

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Chapter 3

ATP-binding cassette transporter gene expression in rat liver progenitor cells

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Submitted

3.1 Abstract

Liver damage results in activation of progenitor cells when proliferation of hepatocytes is suppressed. Progenitor cells are characterized by the expression of specific proteins like Thy-1. We studied the expression of ATP-binding cassette (ABC) transporter genes in rat liver progenitor cells. The progenitor cell compartment was induced by treating rats with 2-acetylaminofluorene (2-AAF) followed by partial hepatectomy (PHx). mRNA levels of ABC transporter genes were determined by real time detection RT-PCR. 2-AAF/PHx-treated animals showed increased hepatic mRNA levels of the genes encoding multidrug resistance protein *Mdr1b* and multidrug resistance protein associated proteins *Mrp1* and *Mrp3*. Immunohistochemistry demonstrated expression of Mrp1 and Mrp3 in progenitor cells and of Mdr1b in periportal hepatocytes. To quantify ABC transporter expression in separate cell types, isolated liver cell fractions were analyzed. Freshly isolated Thy-1 positive cells and cultured RLE φ13 cells, both prototypes for hepatic progenitor cells, highly expressed *Mrp1* and *Mrp3* mRNA, while the hepatocyte-specific transporters *Mdr2*, *Bsep*, *Mrp2*, and *Mrp6* were minimally expressed. The expression pattern of ABC transporters resembled that of cholangiocytes except for *Abca1*, which was expressed in Thy-1 positive cells and RLE φ13 cells, but not in cholangiocytes. In conclusion, as for ABC transporters, hepatic progenitor cells bear more resemblance to cholangiocytes than to hepatocytes. The high expression of Mrp1 and Mrp3 may serve a cytoprotective role in these cells during liver regeneration in conditions of severe hepatotoxicity.

3.2 Introduction

Liver regeneration occurs after loss of liver tissue due to toxic injury or partial hepatectomy (PHx) and involves replication of hepatocytes. Under conditions in which hepatocytes cannot proliferate, liver damage results in the activation of the oval cell compartment.[1] In rats, oval cell proliferation can be achieved by treatment with 2-acetylaminofluorene (2-AAF) in combination with PHx or CCl_4 administration. Treatment with 2-AAF followed by PHx results in a decreased expression of Cyclin E and an increased expression of p53 and p21 in hepatocytes. This causes a block in hepatocyte proliferation at the G1/S restriction point.[2] Oval cells are derived from progenitor cells located in the canals of Hering.[3] These cells escape from the 2-AAF-induced blockade and proliferate in the 2-AAF/PHx model. After hepatic injury, their proliferation reaches a maximal level at 9-11 days post hepatic injury.[4,5] Oval cells resemble fetal hepatic cells as they express fetal markers such as α-fetoprotein and Thy-1 at high levels[4–6] and are considered to be precursors of hepatocytes as well as cholangiocytes.[1,7,8]

Oval cells are only activated after severe liver damage and are supposed to represent a back-up mechanism for hepatic regeneration. We hypothesize that, in view of their critical role in hepatic repair after excessive damage, oval cells should be able to protect themselves against toxic metabolites and xenobiotics. One mechanism of cellular protection could be the expression of efflux pumps that belong to the ATP-binding cassette (ABC) superfamily of membrane transporters.

The ABC transporter family consists of approximately 50 members that have been divided in 7 subclasses (A-G), based on their amino acid sequence homologies. Members of the Abcb (multidrug resistance proteins) subfamily that are expressed in the liver include Mdr1a/b (gene symbol *Abcb1a/b*, MDR1 in humans), efflux pumps for hydrophobic compounds and chemotherapeutic agents, Mdr2 (*Abcb4*, MDR3 in humans), a flippase that translocates phosphatidylcholine across the membrane, and Bsep (*Abcb11*), the export pump for bile salts. These transporters are all located in the canalicular membrane of hepatocytes (reviewed in Hooiveld *et al.*[9]).

The Abcc (multidrug resistance-associated protein) subfamily consists of 12 members, of which at least four, Mrp1, Mrp2, Mrp3, and Mrp6, are expressed in normal liver (reviewed by Borst and Oude Elferink[10]). Mrp2 (*Abcc2*) is present in the apical membrane of hepatocytes.[11] Mrp1 (*Abcc1*) is located in the basolateral membrane but is expressed only at low levels in normal liver. Mrp1 expression is induced in proliferating hepatocytes.[12] Mrp1 and Mrp2 have a similar substrate specificity and transport glutathione S-conjugates, cysteinyl leukotrienes, bilirubin glucuronides, estrogen glucuronides and glutathione disulfide (GSSG).[13,14] Hepatic expression of Mrp3, Mrp4, and Mrp6 has been reported recently.[15] In normal liver, Mrp3 (*Abcc3*) is expressed in the basolateral membrane of pericentrally-localized hepatocytes and in cholangiocytes.[16,17] Mrp3 is able to transport mono- and bivalent bile salts as well as glucuronide conjugates.[18,19] Its expression is increased during cholestasis and in conditions of conjugated or unconjugated hyperbilirubinemia (Gunn rats and Mrp2-deficient mutant rats).[16,20] Mrp4 (*Abcc4*) is an export pump for organic anions, but is also capable of transporting cyclic nucleotides and nucleotide analogues.[21,22]

In normal liver, Mrp4 is expressed at low level.[15] However, enhanced hepatocellular concentrations of bile salts increase Mrp4 expression in liver.[23] Mrp6 (*Abcc6*) is expressed at high levels in normal liver, predominantly in the basolateral membrane of hepatocytes.[24] Its transport specificity is less well defined.

A number of these transporters have been linked to cellular protection. Mdr1b functions as efflux pump for toxins, but may also have an anti-apoptotic role.[25,26] Mrp1 is a transporter of leukotriene C_4 and the glutathione conjugate of prostaglandin A2, factors involved in inflammation and cell cycle arrest. Moreover, Mrp1-mediated transport of glutathione disulfide and glutathione conjugated 4-hydroxynonenal suggests that Mrp1 functions as part of the cellular defense system against oxidative stress (reviewed by Renes *et al.*[13]). Mrp3 may have an important role in protecting cells from endogenous bile salts by extruding these into the blood.[18,19]

Two other members of the ABC family that may be of special interest in relation to oval cells are Abca1 and Bcrp (*Abcg2*). Abca1 is highly expressed in fetal liver, where its expression correlates with apoptotic areas.[27,28] Moreover, Abca1 is ubiquitously expressed in various tissues and organs.[29] It is essential for HDL formation.[30] Its specific function in adult liver is currently unknown. Bcrp is expressed at low levels in the apical membrane of hepatocytes.[31] Expression of Bcrp can, like Mdr1a/b and Mrp1, cause multidrug resistance.[32] Bcrp may have a role in maintaining a dedifferentiated phenotype in a subgroup of hematopoietic stem cells.[33]

We[34] and others[35–37] have studied the expression of a number of these transporters in regenerating rat liver after 70% partial hepatectomy (PHx). In this model mature hepatocytes start to proliferate to make up for the loss of tissue.[38] Twenty-four hours after PHx, at the peak of DNA replication, hepatocytes show a striking increase in *Mdr1b* mRNA expression. *Mdr2* and *Mrp1* are slightly increased after PHx.

The expression profile of ABC transporters in the oval cell compartment is not known. Since that oval cells represent a potential proliferative reservoir of a severely damaged liver, we speculate that these cells must be well protected. We therefore studied ABC transporter expression in hepatic oval cells.

3.3 Materials and methods

Animals

Specified pathogen-free male Wistar and Fisher 344 rats (130-170 g) were purchased from Harlan-CPB, Zeist, the Netherlands, and were kept under routine laboratory conditions with a 12 hour light-dark cycle at the Central Animal Laboratory of the University of Groningen. The rats received standard laboratory chow and had free access to food and water. The Local Committee for Care and Use of Laboratory Animals approved this study.

Animal experiments

2-AAF/PHx-induced oval cell activation in rats

Seven days before PHx, 2-AAF pellets (70 mg/pellet over a 28-day release, 2.5 mg/d, Innovative Research Inc., Sarasota, FL) or placebo pellets were placed subcutaneously in Wistar rats. PHx was performed according to the technique of Higgins and Anderson.[39] Sham-operated animals underwent the same treatment protocol, including manipulation of the intestine and liver, but without hepatectomy. All surgery was performed under halothane anesthesia. Nine days after surgery, livers were perfused with PBS via the portal vein, excised, cut into small pieces and snap-frozen in liquid nitrogen for RNA isolation or frozen in cold 2-methyl-butane for immunohistochemistry. Tissue was stored at -80 °C prior to use.

PHx-induced hepatocyte proliferation in rats

To define transporter expression in proliferating hepatocytes, Wistar rats were either sham operated or received PHx.[39] At 24h post PHx, hepatocytes were isolated using a two-step collagenase perfusion as described before.[40] Cell fractions were frozen in liquid nitrogen prior to RNA isolation.

Isolation of hepatic cell fractions

Specific hepatic cell fractions were isolated from male Fischer 344 rats. Cholangiocytes were isolated using serial counterflow elutriation and isopycnic centrifugation and further purified by immunomagnetic isolation as described by Ishii *et al.*[41] Cells isolated from five rats were pooled to obtain sufficient amounts of RNA. Oval cells were isolated from rats 12 days after 2-AAF/CCl_4-treatment as described by Petersen *et al.*[4] Hepatocytes were isolated as described.[40]

Cell culture

Rat liver epithelial RLE φ13 cells were a kind gift from Dr. S.S. Thorgeirsson, Laboratory of Experimental Carginogenesis, Division of Basic Sciences, National Cancer Institute, Bethesda and have been described previously.[42] The cells were maintained in Ham's F12 (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% FCS and 50 μg/mL gentamycine (Invitrogen Life Technologies). Cells were grown to 90-95% confluence prior to RNA isolation in a humidified incubator at 37°C/5% CO_2.

Antibodies

The mouse monoclonal antibody C219 (Dako, Glostrup, Denmark) was used for detection of all P-glycoproteins (Pgps). This antibody recognizes a conserved epitope close to the ATP-binding cassette in all known members of the P-glycoprotein subfamily.[43,44] The goat polyclonal anti-MRP1 antibody SC-7774 (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection of Mrp1. The epitope detected by anti-MRP1 is located in the amino terminus of human MRP1 and is not conserved in other Mrp family members. The rabbit polyclonal anti-rat Mrp3 antibody was a kind gift from Dr H. Suzuki (University of Tokyo, Tokyo, Japan).[45] OV-6 was a kind gift from Dr S. Sell (Albany Medical College, Albany, NY).[46]

Immunohistochemistry

For immunohistological studies, 4 μm sections were cut from frozen liver tissue. After drying, sections were fixed in acetone for 10 min at room temperature and washed in PBS, immediately before use. Primary antibodies were incubated for 30 min at room temperature. For monoclonal antibodies a peroxidase staining method was used with secondary antibodies, preabsorbed with rat serum (Sigma, St Louis, MO). For the rabbit polyclonal antibodies, the rabbit-EnVision staining method (Dako) was used. For the goat polyclonal antibody, the secondary step consisted of swine anti-goat IgG (Dako), followed by goat peroxidase-anti-peroxidase (Dako). Secondary and tertiary antibodies were diluted in PBS containing 10% normal rat serum. All incubation steps were performed for 30 min at room temperature and were followed by 3 washes in 3 changes of PBS for 5 min.

To evaluate the induction of protein expression, dilution series of the primary antibodies were used (1/10; 1/30; 1/50; 1/100; 1/200; 1/500; 1/1000). Sections from animals from different experimental groups were put together as much as possible on a single slide and different slides were stained in the same batch. When staining was at the limit of detectability and disappeared at a higher dilution, it was evaluated as "critical staining". For each liver specimen, this critical staining was evaluated in each cell compartment separately. By comparing the dilution at which the critical staining was seen, we could evaluate by which factor the protein was up-regulated in each liver cell compartment.

RNA isolation and quantitative PCR

Total RNA was isolated from tissue or cells using TRIzol (Invitrogen Life Technologies) followed by the SV Total RNA isolation system (Promega, Madison, WI) according to manufacturer's instructions. Reverse transcription was performed on up to 5 μg of total RNA using random primers in a final volume of 75 μL (Reverse Transcription System, Promega).

The cDNA levels of the various genes were measured by real-time PCR using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA), a procedure in which cDNA levels are quantitated using a fluorescence signal that is generated during the PCR amplification by cleavage of a fluorogenic probe. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (C_T value) correlate inversely with the mRNA levels.[47] Four microliters of diluted cDNA were used in each PCR reaction in a final volume of 20 μL, containing 900 nM of sense and of antisense primers, 200 nM of fluorogenic probe, 5 mM $MgCl_2$, KCl, Tris-HCl, 0.2 mM dATP, dCTP, dGTP, dTTP, dUTP and 0.5 U of AmpliTaq DNA polymerase (qPCR Core Kit, Eurogentech, Seraing, Belgium). Sequences of the primers and probes used are listed in Table 3.1. Probes were labelled by a 5' FAM (6-carboxy-fluorescein) reporter and a 3' TAMRA (6-carboxy-tetramethyl-rhodamine) quencher. The PCR program was 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was analysed in duplicate. For relative quantification of mRNA expression calibration curves were constructed expressing the log of the input amount as x and C_T as y. *18S* expression levels were used as endogenous control.

Statistics

The data resulting from the experimental groups were expressed as the means ± SD. Differences between the four experimental groups in the 2-AAF/PHx experiment were determined by one-way ANOVA analysis, with posthoc comparison by Student-Newman-Keuls test (SPSS software). An unpaired Student's t test was used to compare the means between the two groups. A P value < 0.05 was considered significant.

3.4 Results

Characteristics of partial hepatectomized rats

Rats were treated either with 2-AAF or placebo for 7 days before undergoing a partial hepatectomy (PHx) or sham operation. The average body weight of the rats was 196 ± 13.1 g when pellets were placed and 242 ± 17.1 g at the time of surgery. There was no significant difference in body weight between rats receiving placebo pellets and rats receiving 2-AAF-containing pellets. On average 7.6 ± 0.7 g of liver were excised during PHx. Animals undergoing 2-AAF/PHx-treatment gained approximately 20 g in weight during the 9 days after PHx, compared to approximately 40 g for rats in the placebo/sham, placebo/PHx, and 2-AAF/sham groups. Liver weights from sham-operated animals were used to calculate the % liver weight/body weight (4.5%). From this, the percentage of liver removed from the partial hepatectomized rats was estimated to be 71%.

ABC transporter gene expression during oval cell activation

2-AAF-treatment without PHx resulted in a mild proliferation of oval cells (Figure 3.1A) as reflected by the presence of more ductuli than seen in normal rat liver (not shown). Massive oval cell proliferation was seen in livers of 2-AAF/PHx treated rats 9 days after PHx (Figure 3.1B).

Figure 3.3 shows the hepatic expression of ABC genes of the four experimental groups, 9 days after PHx or sham operation. The expression of *Mdr1b* mRNA was significantly increased both in 2-AAF and in 2-AAF/PHx-treated rats (79 ± 20 and 120 ± 53 fold relative to 1.0 ± 0.6 and 0.3 ± 0.07 in the placebo/sham and placebo/PHx group). Both *Mdr1a* and *Bsep* mRNA expressions did not significantly change during any of the treatments while *Mdr2* was increased 2-fold in 2-AAF/PHx-treated animals when compared to controls. The expression of *Mrp2* and *Mrp6* was not affected by any treatment, whereas *Mrp1* and *Mrp3* were clearly induced by 2-AAF/PHx-treatment, i.e. approximately 2.5- and 10-fold, respectively. *Mrp4* expression was increased 2-fold under these conditions. *Abca1* and *Bcrp* mRNA expression levels did not change significantly after either treatment.

Immunohistochemical staining

Using immunohistochemistry, the localization of a number of ABC transporters was determined. Figure 3.4A shows the clear canalicular staining pattern of C219, recognizing all P-glycoproteins, in normal rat liver. This C219 staining pattern was much more pronounced in periportal hepatocytes in livers of 2-AAF (not shown) and 2-AAF/PHx-treated

Table 3.1: Sequences of PCR primers and probes used for real-time detection PCR analysis

cDNA		Primers
Mdr1a	sense	5'-GCA GGT TGG CTG GAC AGA TT-3'
	antisense	5'-GGA GCG CAA TTC CAT GGA TA-3'
	probe	5' **FAM**-CCG CCA GAG TTC CCA GCA GCA TG-**TAMRA** 3'
Mdr1b	sense	5'-AAA CAT GGC ACG TAA CCA AAG TT-3'
	antisense	5'-AAA ATG TGG CCC TGT TTA ATG ATT-3'
	probe	5' **FAM**-CAC TGT TAA AGG TAA TTT CAT CAA GAC GAG AAG CCT TC-**TAMRA** 3'
Mdr2	sense	5-AGT TCA CGG GCG CAT CAA-3'
	antisense	5-AAA AGA CAC TGG TGG CAC GTT-3'
	probe	5 **FAM**-CAT CAA GTT CAT TGG TTT CCA CAT CCA GC-**TAMRA** 3'
Bsep	sense	5'-CCA AGC TGC CAA GGA TGC TA-3'
	antisense	5'-CCT TCT CCA ACA AGG GTG TCA-3'
	probe	5' **FAM**-CAT TAT GGC CCT GCC GCA GCA-**TAMRA** 3'
Mrp1	sense	5'-GCC ACT GCC TCA TGC CTA TT-3'
	antisense	5'-GCA AGA CCT GAA GGC AAG ATA CA-3'
	probe	5' **FAM**-AGC CAC ATT TAT AGA GCC AAG CCA GAG CC-**TAMRA** 3'
Mrp2	sense	5'-GAC GAC GAT GAT GGG CTG AT-3'
	antisense	5'-CTT CTC ATG GCC AAG GAA GCT-3'
	probe	5' **FAM**-CCC ACC ATG GAG GAA ATC CCT GAG G-**TAMRA** 3'
Mrp3	sense	5'-TCC CAC TTC TCG GAG ACA GTA ACT-3'
	antisense	5'-CTT AGC ATC ACT GAG GAC CTT GAA-3'
	probe	5' **FAM**-CAG TGT CAT TCG GGC CTA CGG CC-**TAMRA** 3'
Mrp4	sense	5'-TCA GTG TTG GAC AGA GAC AGT TAG TG-3'
	antisense	5'-CTT CTC CCG GAT TTT CTG TTG TAT-3'
	probe	5' **FAM**-TCA GTT CTC GGA TCC ACA TTT GCA GTT G-**TAMRA** 3'
Mrp6	sense	5'-CTC TCC CAT TGG CTT CTT TGA G-3'
	antisense	5'-GTC CAC ATC CAC TAT GTC CGT CT-3'
	probe	5' **FAM**-TCG GGA ACC TGC TGA ACC GTT TTT C-**TAMRA** 3'
Abca1	sense	5'-CCC AGA GCA AAA AGC GAC TC-3'
	antisense	5'-GGT CAT CAT CAC TTT GGT CCT TG -3'
	probe	5' **FAM**-AGA CTA CTC TGT CTC TCA GAC AAC ACT TGA CCA AG-**TAMRA** 3'
Bcrp	sense	5'-CAG GTA GGC AAT TGT GAG GAA GA-3'
	antisense	5'-AAT CAG GGC ATC GAT CTG TCA-3'
	probe	5' **FAM**-CAT GCA AGC CAG GGC CAC ATG A-**TAMRA** 3'
Thy1	sense	5'-GCA GAT GTC CCG AGG ACA GA-3'
	antisense	5'-GGC AGT CCA GTC GAA GGT TCT-3'
	probe	5' **FAM**-CAG CCT GAC AGC CTG CCT GGT GA-**TAMRA** 3'
18S	sense	5'-CGG CTA CCA CAT CCA AGG A-3'
	antisense	5'-CCA ATT ACA GGG CCT CGA AA-3'
	probe	5' **FAM**-CGC GCA AAT TAC CCA CTC CCG A-**TAMRA** 3'

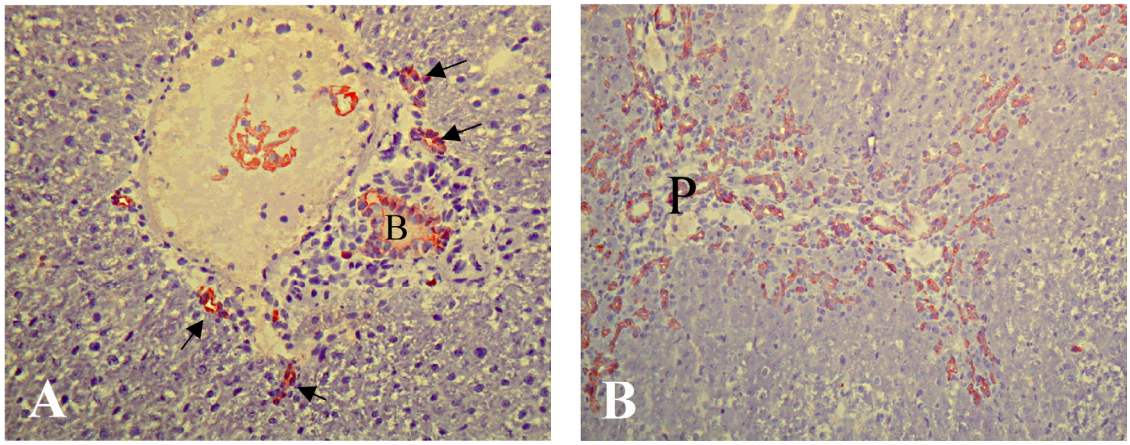

Figure 3.1: Liver sections from rats exposed to 2-AAF only (A) or 2-AAF/PHx (B), obtained at day 9 after surgery. Sections were stained with the OV6 antibody (dilution 1/200). (A) In rats exposed to 2-AAF, OV6 stains the bile duct (right side) and a number of ductuli. (B) In 2-AAF/PHx-treated rats a massively increased number of ductuli is apparent from intensive OV6 staining. B=bile duct; P=portal tract; arrows=oval cell reaction

rats (Figure 3.4B). By diluting the antibody to the level of critical staining, the increase in protein expression for 2-AAF/PHx-treated rats was estimated to be more than 50-fold compared to control.

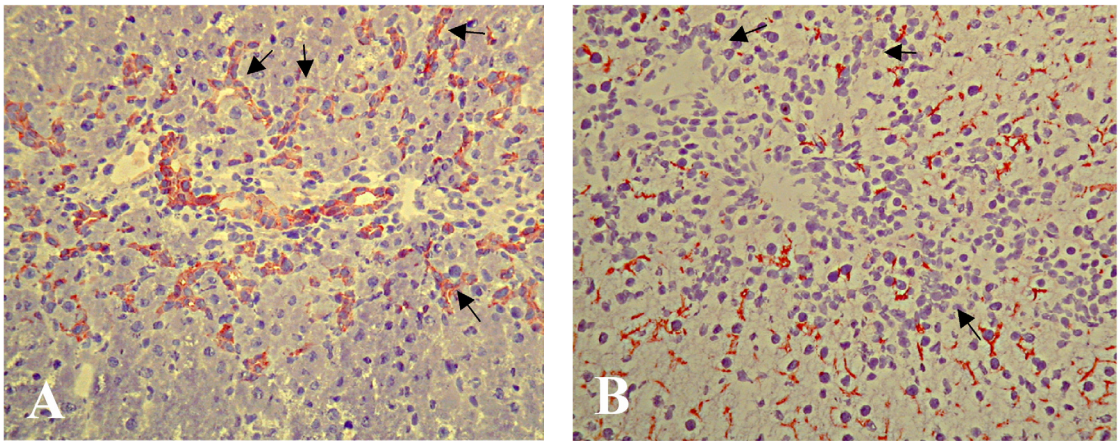

Figure 3.2: Immunohistochemical staining with OV6 (A) and C219 (B) in rat liver 9 days after 2-AAF/PHx-treatment, demonstrating that C219 does not stain the oval cell compartment recognized by OV6. Typical staining patterns of n=3-5 per group. arrows=oval cell reaction

Figure 3.4C shows that the expression of Mrp1 in control rat liver was extremely low. Its expression was increased after 2-AAF/PHx-treatment (Figure 3.4D). By diluting the antibody to the level of critical staining, this increase in protein expression was estimated to be 2 to 5-fold.

The expression of Mrp3 in normal rat liver is shown in Figure 3.4E, with a weak sinusoidal staining of hepatocytes in the centrilobular zone. The bile ducts were also positive as shown in the insert in Figure 3.4E. In livers of 2-AAF/PHx-treated rats (Figure 3.4F) hepatocytes in the entire hepatic acinus became positive, with the strongest signal in perivenous hepatocytes. Mrp3 expression was not increased in cholangiocytes. There was, however, an additional staining of the oval cell compartment after 2-AAF/PHx. This signal was more intense than the signal from the bile ducts.

To further confirm that C219 staining did not localize to the oval cells, staining patterns of OV6 and C219 were compared in serial 4 μm liver sections from 2-AAF/PHx-treated rats. As can be seen in Figure 3.2, no significant co-localization of OV6 (Figure 3.2A) and C219 (Figure 3.2B) was observed.

ABC transporter gene expression in isolated cell fractions

To quantify ABC transporter gene expression in separate cell types, isolated liver cell fractions were analyzed. Thy-1 positive cells were isolated from 2-AAF/CCl_4-treated rats, 12 days after CCl_4 exposure. These cells were compared with cholangiocytes and hepatocytes isolated from normal rats and with the rat liver epithelial cell line RLE φ13. Figure 3.5 shows that Thy-1 positive cells and RLE φ13 cells, both "prototypes" of hepatic progenitor cells, resemble cholangiocytes with respect to ABC transporter gene expression. While normal hepatocytes highly expressed *Mdr2*, *Bsep*, *Mrp2*, and *Mrp6* mRNA, these transporters were virtually absent in Thy-1 positive cells, RLE φ13 cells, and cholangiocytes. However, Thy-1 positive cells and hepatocytes, but not cholangiocytes, expressed high levels of *Abca1* mRNA. Comparison of transporter expression in RLE φ13 with that of Thy-1 positive cells showed that RLE φ13 cells have similar expression characteristics as Thy1-positive cells. The exception in this respect was *Mdr1b* mRNA, which was overexpressed in the RLE φ13 cell line.

ABC transporter gene expression in proliferating hepatocytes

For comparison we also measured the ABC transporter gene expression in proliferating hepatocytes, isolated 24 h after PHx. This time point was chosen because a peak in DNA synthesis, as measured by BrdU incorporation, was observed 24 h after PHx (data not shown). These results are summarized in Table 3.2. Proliferating hepatocytes had a high expression of *Mdr1b* mRNA. The levels of *Mdr2*, *Mrp1*, and *Mrp4* were slightly increased. However, *Mrp3* expression remained unchanged.

3.5 Discussion

In this study we demonstrate that the hepatic mRNA expression of *Mdr1b*, *Mrp1*, and *Mrp3* transporter genes is induced in the 2-AAF/PHx rat model of oval cell proliferation. Immunohistochemistry on livers of 2-AAF/PHx-treated rats showed high expression of Mrp1 and Mrp3 in oval cells. In addition, we show that isolated Thy-1 positive cells and cultured RLE φ13 cells, prototypes of hepatic progenitor cells, both express particular high levels of Mrp3 and Mrp1. This supports the *in vivo* results of the 2-AAF/PHx model. High

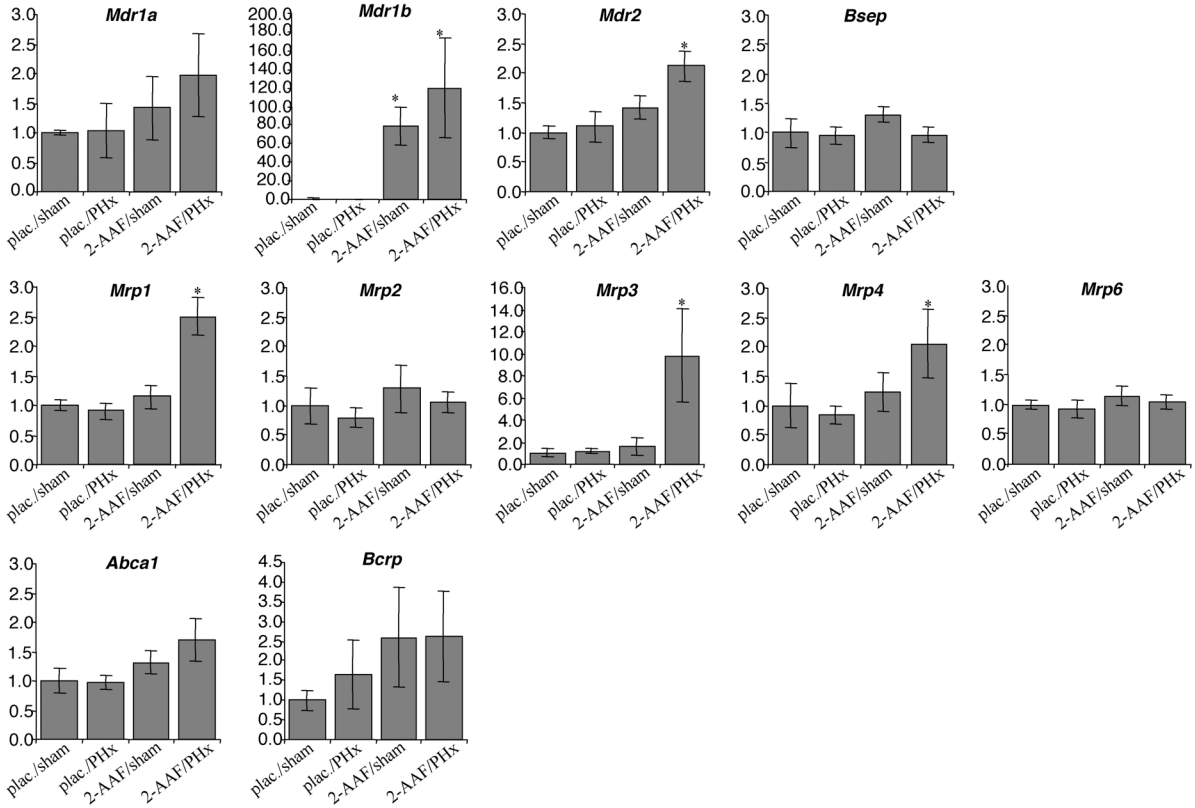

Figure 3.3: mRNA levels of ABC transporter genes in the liver during oval cell activation by 2-AAF/PHx-treatment. Relative mRNA expression levels of various ABC transporter genes were determined by real time detection RT-PCR as described in Materials and methods. plac/sham: placebo pellet followed by sham operation; plac/PHx: placebo pellet followed by PHx; 2-AAF/sham: 2-AAF pellet followed by sham operation; 2-AAF/PHx: 2-AAF pellet followed by PHx. Levels expressed relative to plac./sham levels (set to 1). Data points represent means ± S.D., n=3-5 per group, $^{\star}P < 0.05$.

expression of these transporters suggests that they have a role in cellular protection of this important cell population.

In the model used in this study, proliferation of hepatocytes after PHx was blocked by pre-treatment with 2-AAF. In hepatocytes, 2-AAF is converted via phase I drug metabolism. As 2-AAF or its metabolites may by themselves induce the expression of ABC transporters, one group of animals received 2-AAF alone. These animals showed a mild activation of the oval cell compartment. *In vitro*, 2-AAF has been shown to increase the expression of *Mdr1b* and *Mrp2* in cultured rat hepatocytes and in rat hepatoma cell lines as well as the expression of *MRP2*, *MRP3*, and *MRP5* in the human hepatoma cell line HepG2.[48–50] *In vivo* in rats, 2-AAF has been reported to induce hepatic *Mdr1b* expression.[35,36] Our results confirmed the large increase in *Mdr1b* mRNA expression but the mRNA expression levels of both *Mrp2* and *Mrp3* were found to be not affected by 2-AAF in the *in vivo* situation.

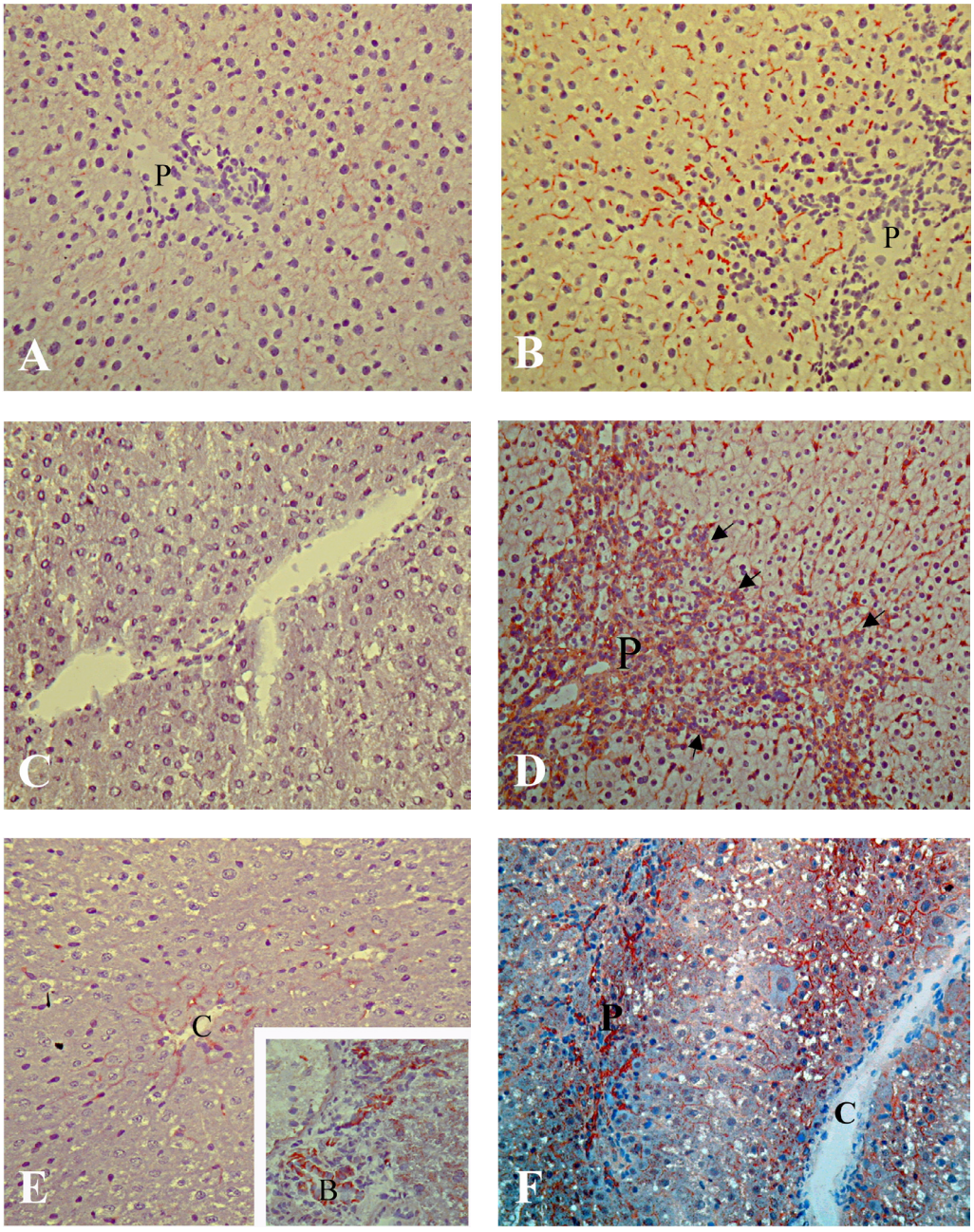

Figure 3.4: Immunohistochemical staining of C219 (A, B), Mrp1 (C, D), and Mrp3 (E, F) in control rat liver (A, C, E) and 2-AAF/PHx-treated rat liver (day 9) (B, D, F). Frozen liver sections were stained with primary antibodies directed against all P-glycoproteins (using C219, dilution 1/5), Mrp1 (SC-7774, dilution 1/5), or Mrp3 (anti-Mrp3, dilution 1/500). In normal liver, C219 weakly stained the canalicular membrane of hepatocytes (A). In treated rat liver C219 staining was increased (B). SC-7774 did not stain normal liver, but co-localized with the oval cell compartment in treated liver (D). In normal liver anti-Mrp3 stained the pericentral hepatocytes (E) and the bile ducts (insert in E). In treated there was additional staining of the oval cells (F). Typical staining patterns of n=3-5 per group. B=bile duct, P=portal tract, C=central vein; arrows=oval cell reaction

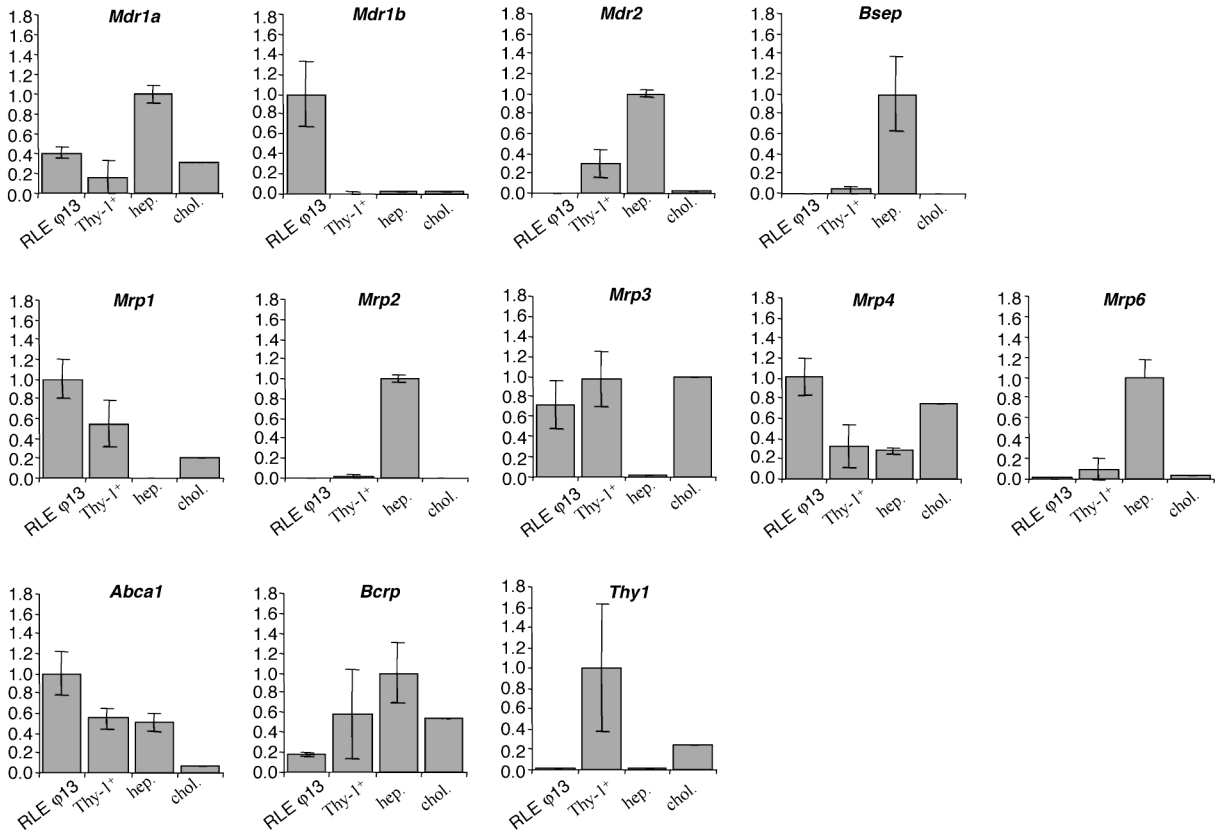

Figure 3.5: mRNA levels of ABC transporter genes in RLE φ13 cells, Thy-1 positive cells (Thy-1+), hepatocytes (hep.), and cholangiocytes (chol.) The relative expression levels (highest level set to 1) of various ABC transporter genes in the different cell fractions were determined by real time detection RT-PCR as described in Materials and methods.

Deng *et al.*[50] have recently elucidated the intracellular signal transduction pathway involved in the up-regulation of *Mdr1b* mRNA levels by 2-AAF in an *in vitro* model. They demonstrated that transcription of *Mdr1b* is induced by NF-κB. The activation of NF-κB is the ultimate result of oxidative stress generated by 2-AAF.[50] Moreover, 2-AAF has been demonstrated to increase p53 expression.[2] p53, in turn, can induce *Mdr1b* expression.[51] The strong C219 staining pattern in rats treated with 2-AAF only can thus, at least in part, be explained by direct effects of 2-AAF on hepatocytes. After 2-AAF/PHx-treatment, when there is extensive oval cell activation, the Mdr1b protein expression seemed to be confined to periportal hepatocytes and did neither occur in the OV-6 positive cell compartment nor in bile duct epithelial cells. This observation contrasts with the human situation. In regeneration after submassive necrosis, there is increased expression of MDR1 both in the remaining hepatocytes and in the regenerating ductules, the latter representing the putative progenitor cells (Ros *et al.*, submitted).

We used the commercially available antibody SC-7774 to study Mrp1 expression. This

Table 3.2: Expression of ABC transporter genes after 70% PHx relative to control as determined by real-time detection PCR analysis

Gene	24 h Sham	24 h PHx
Mdr1a	1.00 ± 0.37	1.29 ± 0.26
Mdr1b	1.00 ± 0.25	39.47 ±1.09*
Mdr2	1.00 ± 0.29	2.08 ± 0.36*
Bsep	1.00 ± 0.29	0.89 ± 0.26
Mrp1	1.00 ± 0.41	1.90 ± 0.20*
Mrp2	1.00 ± 0.41	0.84 ± 0.08
Mrp3	1.00 ± 0.27	1.33 ± 0.39
Mrp4	1.00 ± 0.13	2.15 ± 0.35*
Mrp6	1.00 ± 0.21	0.66 ± 0.36
Abca1	1.00 ± 0.40	1.37 ± 0.23
Bcrp	1.00 ± 0.65	0.69 ± 0.13

antibody has been raised against an Mrp1-specific epitope. In normal liver, SC-7774 staining was extremely weak indicating that SC-7774 does not recognize Mrp2 (located in the canaliculi) or Mrp3 (located in the cholangiocytes). Furthermore, the antibody does not recognize Mrp4, as Mrp4 lacks the extra N-terminal region against which SC-7774 has been raised. Moreover, the extremely low staining in normal liver suggests that SC-7774 does not recognize Mrp6, which is highly expressed in normal liver. Thus, we conclude that SC-7774 is specific for Mrp1. The increase in SC-7774 staining observed in 2-AAF/PHx-treated rats (Figure 3.4D) therefore demonstrates increased Mrp1 expression.

To further define the expression profiles of ABC transporters in different cell types, we compared isolated liver cell fractions. We also compared Thy-1 positive cells with the liver epithelial cell line RLE φ13, which resembles oval cells, by expressing factors as c-kit, OV-6 and α-fetoprotein.[42] Compared to Thy-1 positive cells, RLE φ13 cells were found to have a highly increased expression of *Mdr1b*, a phenomenon that is frequently observed with cultured cells.[52] Levels of other ABC transporters also change during culture. When comparing freshly isolated cells with cultured cells (hepatocytes with cultured hepatocytes and rat hepatoma cells [H4IIE cell line]; Thy-1 positive cells with cultured RLE φ13 cells; and freshly isolated cholangiocytes with cultured NRC cells[53]) we consistently observed an increase in *Mdr1a*, *Mdr1b* and *Mrp1* mRNA expression during culture. Conversely, expression levels of the hepatocellular transporters *Mdr2*, *Bsep*, *Mrp2*, and *Mrp6* decreased during culture (own observations, as well as [54,55]). *Mrp3* expression increased in most cultured cells, but the already high *Mrp3* expression of Thy-1 positive cells was not further increased in RLE φ13 cells. Despite these conditional changes, the expression pattern of ABC transporters in Thy-1 positive cells is largely retained in RLE φ13 cells in that they both have high mRNA levels of *Mrp1* and *Mrp3* and low mRNA levels of *Mdr2*, *Bsep*, *Mrp2*, and *Mrp6*.

There was a remarkable difference between Thy-1 positive cells and cholangiocytes in expression of *Abca1*, which was found to be expressed in Thy-1 positive cells but not

in cholangiocytes. The function of Abca1 in oval cells is not yet known. It may promote engulfment of apoptotic cells by translocating phosphatidylserine to the outer plasma membrane leaflet,[27] which would correlate with the high prevalence of apoptosis in the oval cells compartment.[56]

It has recently been shown that primitive hematopoietic stem cells highly express *Bcrp*.[33] As hepatic oval cells display primitive features, we speculated that these cells also have a high *Bcrp* expression. However, in the 2-AAF/PHx model, *Bcrp* mRNA expression was not induced. Moreover, *Bcrp* mRNA expression levels were similar in the different cell fractions studied. It may be that Thy-1 positive cells have already acquired a more differentiated phenotype and have lost *Bcrp* expression.

This study demonstrates that hepatic oval cells have high expression levels of Mrp1 and Mrp3. Proliferating hepatocytes, isolated 24 h after PHx, expressed highly increased levels of *Mdr1b* whereas *Mdr2*, *Mrp1*, and *Mrp4* were increased to a lesser extent (Table 3.2, [34]). *Mrp3* expression was not increased in proliferating hepatocytes after PHx. Thus, *Mrp3* up-regulation mainly occurs in oval cells, whereas *Mdr1b* up-regulation mainly takes place in hepatocytes.

In conclusion, our findings show that the up-regulation of Mdr1b in the 2-AAF/PHx model is confined to hepatocytes while the expression of Mrp1 and Mrp3 mainly occurs in the oval cell compartment. Hepatic oval cells and cholangiocytes bear a close resemblance as far as their ABC transporter expression is concerned. Both cell types appear to be well protected by ABC transporters, which may enable these cells to survive conditions associated with excessive metabolic stress and serve as a proliferative reservoir when hepatocytes are severely damaged.

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Chapter 4

High expression of MDR1, MRP1, and MRP3 in the hepatic progenitor cell compartment and hepatocytes in severe human liver disease

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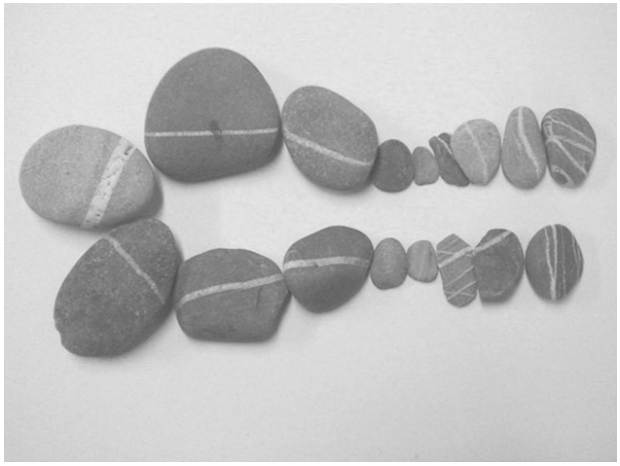

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Submitted

4.1 Abstract

In diverse human liver diseases an increase in bile ductular structures is observed. These structures harbour the progenitor cell compartment of the liver. Since ATP-binding cassette transporters may serve a cytoprotective role in liver disease, we performed an immunohistochemical study on human liver specimens of patients with submassive cell necrosis, chronic hepatitis C (HCV), primary biliary cirrhosis (PBC), and normal liver. The expressions of MDR1, MDR3, BSEP, MRP1, MRP2, and MRP3 were determined using specific antibodies. Dilution series were made until the critical staining level, to semiquantify the factor of up-regulation. In normal liver, hepatocytes showed a canalicular staining for MDR3, BSEP, and MRP2. MDR1 stained the canalicular membrane of the hepatocytes as well as of the cholangiocytes. MRP3 showed low immunoreactivity of the bile duct epithelial cells and of the pericentral hepatocytes only. Normal liver was not immunoreactive for MRP1. In diseased liver, the expression of MDR3, BSEP, and MRP2 was relatively stable. Only during severe cholestasis, the expression of BSEP and MRP2 decreased. The expression levels of MDR1, MRP1, and MRP3, however, were increased in PBC, HCV, or submassive necrosis. Strongest immunoreactivity was seen after submassive necrosis, where remaining islands of hepatocytes showed strong canalicular staining for MDR1 (20-50-fold up-regulation) and MRP3 (20-fold up-regulation). The expression of MRP1 in remaining hepatocytes was heterogeneous. Regenerating bile ductules at the interface of portal tracts and necrotic areas intensely stained for MDR1, MRP1, and MRP3. In conclusion, MDR1, MRP1, and MRP3 are up-regulated in hepatocytes in severe human liver disease. Strong MDR1-, MRP1-, and MRP3-reactivity is seen in human regenerating bile ductules.

4.2 Introduction

In various human and rodent liver diseases a striking increase in the number of bile ductular structures is observed. These form a labyrinth at the edge of the portal tracts (ductular reaction). These structures harbour the progenitor cell compartment of the liver.[1]

Hepatic progenitor cells are activated after liver damage and have a critical role in hepatic repair after excessive damage.[1–4] We therefore hypothesize that these cells should be able to protect themselves against toxic metabolites and xenobiotics. One mechanism of cellular protection could be the expression of efflux pumps that belong to the ATP-binding cassette (ABC) superfamily of membrane transporters.

In normal liver ABC transporters participate in diverse cellular processes, mediating excretion of bile salts, organic anions, lipids or xenobiotics. This way they contribute to bile formation and enable cells to keep the intracellular levels of toxic compounds low.

The ABC transporter family has been divided in 7 subclasses (A-G) (reviewed by Klein *et al.*[5] and Borst and Oude Elferink[6]). Members of the ABCB and ABCC subfamilies are important for bile formation. Members of the ABCB subfamily that are expressed in the liver include MDR1 (gene symbol *ABCB1*), an efflux pump for hydrophobic compounds, MDR3 (*ABCB4*), a translocator of phosphatidylcholine across the membrane, and BSEP (*ABCB11*), the export pump for bile salts. These transporters are all located in the apical membrane of hepatocytes.

The ABCC subfamily consists of 12 members, of which five have now been identified in liver tissue. Best characterised are the apical multidrug resistance related protein 2 (MRP2, *ABCC2*) and its basolateral homologues MRP1 (*ABCC1*) and MRP3 (*ABCC3*). MRP2 transports amphiphilic anionic conjugates into bile. MRP1 transports the same substrates, but its expression levels are very low in normal liver. MRP3 is able to transport organic anions, including bile salts, into blood.[7,8] Expression levels of MRP2 and MRP3 appear to be counter-regulated: when MRP2 levels decrease, an increase in MRP3 expression is observed.[9,10] This provides the cell with a compensatory mechanism for secretion of bile salts under cholestatic conditions. In addition to its expression in hepatocytes, MRP3 has been identified in the basolateral membrane of cholangiocytes.[11]

We have previously studied ABC transporter gene expression in a rat model of hepatic progenitor cell activation. These rats were treated with 2-acetylaminofluorene (2-AAF) prior to partial hepatectomy. We observed a high expression of Mrp1 and Mrp3 in the progenitor cells in these rats (Ros *et al.*, submitted).

Here we aimed to study ABC transporter gene expression in human liver with progenitor cell activation. To study the different human liver cell compartments separately, we performed immunohistochemical analyses using specific antibodies against ABC transporter molecules. To evaluate up- or down-regulation of the transporter proteins in the different segments of the bile duct system and the hepatocytes, we made dilution series of the primary antibodies until the staining disappeared in the different cell compartments.

4.3 Materials and methods

Immunohistochemistry

Forty human liver specimens taken from liver explants prior to liver transplantation or needle biopsies taken for diagnostic purposes, were used for this study: normal liver (n=5), PBC (n=10), HCV (n=15) and regeneration after submassive liver necrosis (n=10). From each specimen, part was snap-frozen in liquid nitrogen-cooled isopentane, part was fixed in B5 fixative or in formaldehyde and embedded in paraffin for routine diagnosis, and part was fixed for electron microscopy. The diagnosis was made on the basis of examination of the paraffin-embedded material, clinical and laboratory data. Informed consent was obtained from all patients and the experimental protocol was approved by the Ethical Committee of the University Hospital of Leuven, in accordance with the Ethical Guidelines of the 1975 Declaration of Helsinki.

Normal liver biopsies showed a preserved architecture, no inflammation, no steatosis, and no cholestasis. Biopsies from patients with PBC showed advanced portal-portal septum formation in 8 patients and established cirrhosis in 2 patients. There was advanced ductopenia and a marked ductular reaction at the periphery of the portal tracts and septa. A marked degree of portal inflammation and granuloma formation was seen. Periportal and periseptal hepatocytes showed signs of cholate stasis with swelling and a pale aspect of the cytoplasm, sometimes with formation of cholestatic Mallory bodies. Cholestatic liver cell rosettes and bilirubinostasis were also present.

Biopsies of chronic HCV patients showed a varying degree of fibrosis ranging from periportal fibrosis (n=5) over septal fibrosis (n=4) to cirrhosis (n=6). The portal tracts showed varying degrees of mononuclear inflammatory infiltrate with lymphoid aggregate formation and interface hepatitis. Also lobular/nodular inflammation was variable.

In specimens with submassive liver cell necrosis, large areas of parenchyma had disappeared, leaving only islands of remaining hepatocytes. High numbers of ceroid macrophages were seen. At the edges of portal tracts, regenerating ductules were present. The degree of infiltrate in the portal tracts and necrotic areas varied according to the cause of necrosis. In the cases of toxic liver cell necrosis the infiltrate was moderately dense and contained a relatively high number of eosinophilic polymorphonuclear leucocytes. In the cases of viral hepatitis, the lymphocytic infiltrate was very marked. In the cases of auto-immune hepatitis, lymphocytes were intermingled with large clusters of plasma cells.

Immunohistochemistry was performed on frozen sections. Five micrometers thick cryostat sections were dried overnight, were subsequently fixed in acetone for 10 minutes and finally washed in PBS, immediately before use. All samples were incubated with the primary antibody for 30 min at room temperature. The primary antibodies used are listed in Table 4.1. P_3II-26, M_2I-4 and M_3II-9 were kindly provided by Dr. R.J. Scheper, Free University, Amsterdam, the Netherlands. JSB1 was obtained from Alexis Biochemicals (Lausen, Switzerland). SC-7774 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). OV-6 was a kind gift from Dr. S. Sell (Albany Medical College, Albany, NY). Anticytokeratin 7 and 19 were obtained from Dako (Glostrup, Denmark) and from Amersham Life Sciences (Amersham, UK), respectively. With all rabbit polyclonal antibodies, a two-step

Table 4.1: List of antibodies used

	name	species	reference
MDR1	JSB1	mouse mAB	Scheper *et al.*[20]
MDR3	P_3II-26	mouse mAB	Scheffer *et al.*[21]
BSEP	k12	rabbit pAB	Jansen *et al.*[22]
MRP1	SC 7774	goat pAB	
MRP2	M_2I-4	mouse mAB	Scheffer *et al.*[21]
MRP3	M_3II-9	mouse mAB	Scheffer *et al.*[21]

peroxidase-labeled method was used. Slides were blocked with normal goat serum (dilution 1/5 in PBS) for 7 min, followed by incubation with the primary antibody for 30 min at room temperature. Subsequently, slides were incubated with undiluted Rabbit-EnVision (Dako) for 30 min at room temperature. With the goat polyclonal antibodies, the second step consisted of swine anti-goat IgG (Dako), followed by goat peroxidase-anti-peroxidase (Dako). With all monoclonal antibodies, a three-step indirect immunoperoxidase procedure was used: a peroxidase-labeled rabbit anti-mouse IgG (Dako) was followed by peroxidase-labeled swine anti-rabbit IgG (Dako). All secondary and tertiary antibodies were diluted in PBS containing 10% normal human serum. All incubation steps were followed by a wash in three changes of PBS for 5 min. The reaction product was developed with the use of 3-amino-9-ethylcarbazole and H_2O_2 (0.01%) and finally the sections were counterstained with haematoxylin. Controls consisted of omission of the primary antibody or replacement by normal rabbit immunoglobulins (Dako) or normal mouse ascites (Dako) and showed absence of specific staining.

To evaluate the factor up-regulation of the proteins, dilution series of the primary antibodies were used (1/5; 1/10; 1/20; 1/30; 1/50; 1/100; 1/200; 1/300; 1/500; 1/1000). Four to five frozen sections were put together on one slide in order to stain all sections in the same conditions. When a staining was at the limit of detectability and disappeared in a higher dilution, we evaluated this as "critical staining". For each liver specimen this critical staining was evaluated in each cell compartment separately (hepatocytes, ductules, interlobular ducts). By comparing the dilution at which the critical staining was seen, we could evaluate with which factor the protein was up-regulated in each liver cell compartment. Evaluations of the stainings were done by two independent pathologists in a blinded way. The slides were mixed up randomly and coded by an independent person.

RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissue using TRIzol (Invitrogen Life Technologies, Breda, the Netherlands) according to manufacturers instructions. Reverse transcription was performed on 3.5 μg of total RNA using random primers in a final volume of 75 μL (Reverse Transcription System, Promega, Madison, WI).

The cDNA levels of the various genes were measured by real-time polymerase chain reaction (PCR) using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) where cDNA levels are quantitated using a fluorescence signal that is generated

Table 4.2: Sequences of PCR primers and probes used for real-time detection PCR analysis

cDNA		Primers
MDR1	sense	5'-GGC AAA GAA ATA AAG CGA CTG AA-3'
	antisense	5'-GGC TGT TGT CTC CAT AGG CAA T-3'
	probe	5' **FAM**-CGT GTC CCA GGA GCC CAT CCT GT-**TAMRA** 3'
MDR3	sense	5'-TCA ATG GCT TTT AAA GCA ATG CTA-3'
	antisense	5'-TGC AAT TAA AGC CAA CCT GGT T-3'
	probe	5' **FAM**-CAC AGA TGC TGC CCA AGT CCA AGG A-**TAMRA** 3'
BSEP	sense	5-ACA TGC TTG CGA GGA CCT TTA-3'
	antisense	5-GGA GGT TCG TGC ACC AGG TA-3'
	probe	5 **FAM**-CCA TCC GGC AAC GCT CCA AGT CT-**TAMRA** 3'
MRP1	sense	5'-CTT CTG GAG GAA TTG GTT GTA TAG AAG-3'
	antisense	5'-GGT AGA CCC AGA CAA GGA TGT TAG A-3'
	probe	5' **FAM**-TCT TTG AGA TGC TTC TGG CTC CCA TCA C-**TAMRA** 3'
MRP2	sense	5'-TGC AGC CTC CAT AAC CAT GAG-3'
	antisense	5'-CTT CGT CTT CCT TCA GGC TAT TCA-3'
	probe	5' **FAM**-CAG CTT TCG TCG AAC ACT TAG CCG CA-**TAMRA** 3'
MRP3	sense	5'-GCC ATC GAC CTG GAG ACT GA-3'
	antisense	5'-GAC CCT GGT GTA GTC CAT GAT AGT G-3'
	probe	5' **FAM**-CAT CCG CAC CCA GTT TGA TAC CTG CAC-**TAMRA** 3'
18S	sense	5'-CGG CTA CCA CAT CCA AGG A-3'
	antisense	5'-CCA ATT ACA GGG CCT CGA AA-3'
	probe	5' **FAM**-CGC GCA AAT TAC CCA CTC CCG A-**TAMRA** 3'

during the PCR amplification by cleavage of a fluorogenic probe. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (C_T value) correlate inversely with the mRNA levels.[12] Four microliters of diluted cDNA were used in each PCR reaction in a final volume of 20 μL, containing 900 nM of sense and of antisense primers, 200 nM of fluorogenic probe, 5 mM $MgCl_2$, KCl, Tris-HCl, 0.2 mM dATP, dCTP, dGTP, dTTP, dUTP, and 0.5 U of AmpliTaq DNA polymerase (qPCR Core Kit, Eurogentech, Seraing, Belgium). Sequences of the primers and probes used are listed in Table 4.2. Probes were labelled by a 5' FAM (6-carboxy-fluorescein) reporter and a 3' TAMRA (6-carboxy-tetramethyl-rhodamine) quencher. The PCR program was 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Each sample was analysed in duplicate. For relative quantification of mRNA expression calibration curves were constructed expressing the log of the input amount as x and C_T as y. *18S* expression levels were used as endogenous control.

4.4 Results

Enhanced expression of MDR1, MRP1, and MRP3 protein in reactive bile ductules and hepatocytes

In **normal** liver, **MDR1** showed a canalicular staining pattern in hepatocytes. At a dilution of 1/10, this hepatocyte canalicular staining was at the "critical staining" level. The interlobular bile ducts showed apical reactivity, which was at the critical staining level at a diltution of 1/30 (Figure 4.1A, Table 4.3). In **advanced PBC**, MDR1 showed strong canalicular staining at a dilution of 1/10. The critical staining level was 1/50. This means a factor 5 up-regulation compared to normal. Reactive ductules, identified by their immunoreactivity for OV-6, cytokeratin 7 and 19, were strongly immunoreactive at the apical pole at a 1/10 dilution with a critical staining level of 1/50. In **chronic HCV**, canalicular staining of MDR1 was up-regulated with a factor 20-30 (critical staining at a dilution of 1/200-1/300). The stronger up-regulation was seen in severely active hepatitis, as well in early stages of fibrosis as in the cirrhotic stage. Reactive ductules showed very strong apical reactivity, with a critical staining of 1/300 to 1/1000, the strongest up-regulation seen in liver biopsies with severely active hepatitis (Figure 4.1B). In **regeneration after submassive necrosis** up-regulation of canalicular reactivity of MDR1 with a factor 20-50 was seen in islands of remaining hepatocytes, while regenerating ductules/progenitor cells showed strong reactivity which was also still recognizable at dilution 1/200-1/500 (Figures 4.1C, D).

MRP1 could not be detected in **normal** human liver. In **advanced PBC, chronic HCV**, and **submassive necrosis**, hepatocytes were heterogeneously stained at a dilution of 1/5-1/10. Some interlobular bile ducts were weakly positive at a dilution of 1/5, while some were negative at this dilution. In **severely active hepatitis** as well as in **regeneration after submassive necrosis**, reactive ductules showed strong reactivity, with a critical staining level at a dilution of 1/10 (Figure 4.1H).

In **normal** human liver, **MRP3** showed basolateral hepatocyte reactivity in 2-3 layers of hepatocytes surrounding the central vein and in the interlobular bile ducts at a dilution of 1/10. This staining had already disappeared at a dilution of 1/30 (Figure 4.1E). In **advanced PBC**, basolateral hepatocytic staining in the centrolobular zone was still present at a dilution of 1/100. In addition, periportal hepatocytes were reactive. In cirrhotic end-stage PBC, diffuse nodular hepatocyte staining was present at a dilution of 1/10, 1/30 and 1/50, but disappeared at dilution 1/100. Basolateral ductular reactivity in the majority of bile ductules was still visible at dilution 1/100. Remaining interlobular bile ducts were negative at this dilution. This means an overall up-regulation with a factor 10 in centrolobular, and periportal hepatocytes and in reactive bile ductules in advanced PBC, while interlobular bile ducts showed no (or less) up-regulation (Figure 4.1F). In **chronic HCV**, the degree of MRP3 up-regulation was less pronounced in specimens with little inflammatory activity. In liver specimens with severely active hepatitis, basolateral hepatocyte reactivity was seen throughout the lobule/cirrhotic nodule. The up-regulation varied from a factor 2 to 20, depending on the degree of inflammation. Regenerating ductules showed strong reactivity at a dilution of 1/10, and critical staining at a dilution of 1/50. This means a

Table 4.3: Overview of antibody dilutions for critical staining for MDR1 and MRP3

	normal liver		advanced PBC		chronic hepatitis C		submassive necrosis	
	hepatocyte	ILBD	hepatocyte	ductules	hepatocyte	ductules	hepatocyte	ductules
MDR1	1/10	1/30	1/50	1/50	1/200-1/300	1/300-1/1000	1/200-1/500	1/200-1/500
MRP1	n.d.	n.d.	1/5-1/10	1/5-1/10	1/5-1/10	1/5-1/10	1/5-1/10	1/5-1/10
MRP3	CL 1/10	1/10	CL 1/100	1/100	1/20-1/200	1/50	1/200	1/50-1/100

ILBD: Interlobular bile ducts, n.d.: not detectable, CL centrolobular

factor 5 up-regulation. In **regeneration after submassive necrosis** remaining islands of hepatocytes showed diffuse basolateral reactivity for MRP3, up-regulated with a factor 20. Regenerating ductules showed strong reactivity at dilution 1/10 and critical staining at dilution 1/50 and for a subset of ductules at dilution 1/100 (Figure 4.1G). The results of these dilution series are summarized in Table 4.3.

MDR3, BSEP, and MRP2 expression remains relatively stable during hepatic disease

MDR3, BSEP, and MRP2 showed a canalicular staining pattern of hepatocytes, while bile ducts and ductules were negative. No clear differences in expression were seen between normal liver, PBC, chronic hepatitis, or submassive necrosis. Only in end-stage PBC, scattered hepatocytes with extreme cholate stasis, containing cholestatic Mallory bodies, showed down-regulation of MRP2 and to a lesser extent of BSEP.

RT-PCR

To strengthen the results obtained by immunohistochemistry, we performed quantitative RT-PCR to determine mRNA expression levels of a number of transporters. RNA was isolated from liver biopsies, and only from a limited number of samples sufficient RNA was obtained (4 controls, 3 submassive necrosis, and 3 HCV patients). These results are shown in Figure 4.2. Although levels of transporters were highly variable among the samples, and the groups are small, some trends could be observed. In total liver, *MDR1* expression levels had a tendency to increase. Likewise, *MRP1* and *MRP3* levels increased to some extent. *BSEP* and *MRP2* mRNA expression was relatively stable, with one exception: one patient with HCV had a high *BSEP* mRNA expression.

4.5 Discussion

Since ABC transporters play both a secretory and a protective role, we studied the expression and regulation of ABC transporters in different human liver cell compartments (hepatocytes, ductular/progenitor cell compartment, interlobular bile ducts) in normal and diseased human liver, using immunohistochemistry and dilution series. The results were further strengthened by RT-PCR.

In normal human liver, MRP3 was expressed in 2-3 layers of centrolobular hepatocytes, as is seen in the rat.[10,13] The function of this export pump could be the centrolobular secretion of glucuronides into the circulation, since these are predominantly formed in the

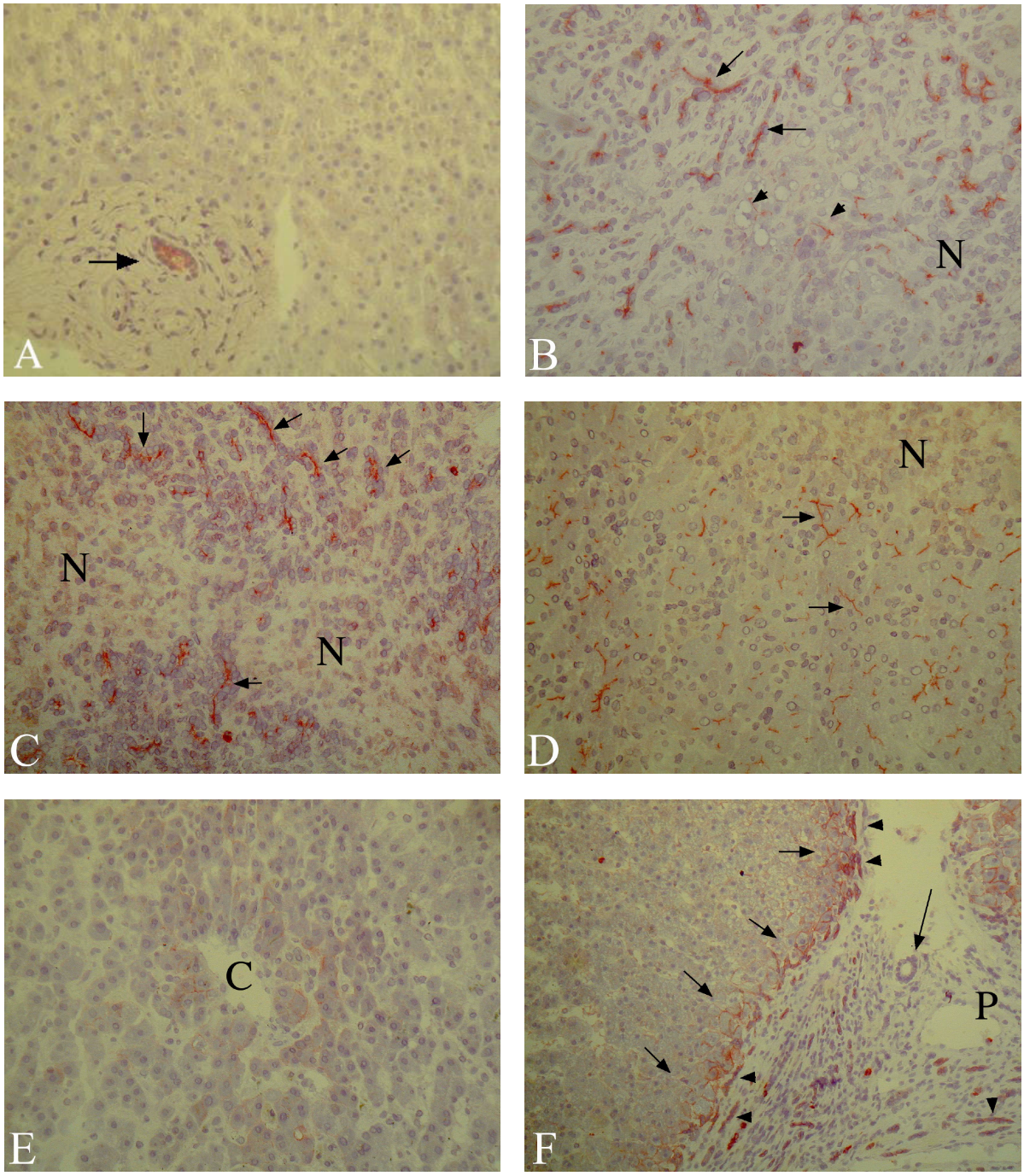
pericentral hepatocytes.[14] In chronic cholestasis (PBC), periportal hepatocytes became immunoreactive for MRP3. In the cirrhotic stage, some nodules showed diffuse MRP3 reactivity, in accordance to what is seen after long-term bile duct ligation in the rat.[13] This up-regulation probably serves as a protective mechanism against the accumulation of toxic bile salts in hepatocytes. In contrast, the canalicular organic anion transporter MRP2 showed a down-regulation in severely cholestatic hepatocytes, while BSEP expression was decreased in severe cholestatic hepatocytes in some patients, but remained stable in other patients. The fact that these canalicular transporters were only down-regulated in hepatocytes with severe cholestasis, containing cholestatic Mallory bodies, suggests that these transporters are maintained in their location and perfom their function for a long time, even in cholestatic livers. MRP2 and MRP3 have overlapping substrate specificity,[7,8] so the apical down-regulation of MRP2 and the basolateral up-regulation of MRP3 in periportal cholestatic hepatocytes probably represents an adaptation mechanism which protects hepatocytes against accumulation of toxic bile constituents.

MDR1 and MRP3 were expressed in normal bile ducts, suggesting a functional role for these transporters in normal bile formation in both interlobular bile ducts and ductules. The cholangiocellular expression of MDR1 and MRP3 in normal human liver specimens was stronger than the canalicular MDR1 and basolateral MRP3 expression in hepatocytes. MDR1 and MRP3 were both up-regulated in reactive bile ductules/progenitor cells.

In chronic cholestatic liver diseases, reactive ductules form a labyrinth at the edge of the portal tracts and are thought to form a reservoir in which toxic bile can accumulate.[1,15] The up-regulation of MDR1 and MRP3 in ductular structures in PBC probably serves as a protective measure against the accumulation of toxic bile constituents. The fact that reactive bile ductules close to the parenchyma showed higher reactivity for MRP3 compared to ductules deeper in the portal tract and interlobular bile ducts supports this hypothesis. MRP3 probably functions as basolateral transporter to extrude bile salts back to the systemic circulation, thereby contributing to cholehepatic shunting.

In conditions of hepatocyte injury and loss, like in hepatitis, ductular reaction contributes to hepatocyte regeneration. Reactive bile ductules are at least partly the result of activation, proliferation, and differentiation of bipotential progenitor cells, which can differentiate towards hepatocytes and bile duct epithelial cells. The striking up-regulation (factor 20-50) of MDR1 and to a lesser extent of MRP3 (factor 5 in regenerating ductules, factor 2-20 in hepatocytes) and MRP1 in this cell compartment and in remaining hepatocytes could provide these cells with a multidrug-resistent phenotype. It has been demonstrated that MDR1 has anti-apoptotic effects.[16,17] Via MRP1, cells can secrete products of oxidative stress reactions, such as GSSG or the GSH conjugates of 4-hydroxynonenal.[18] This could be especially relevant for the progenitor cell compartment and the remaining islands of hepatocytes. The up-regulation of MDR1, MRP1, and MRP3 was seen in viral hepatitis and in submassive liver cell necrosis of different etiologies, suggesting that this may be a general protective mechanism, rather than a toxin-induced effect.

We aimed to quantify the mRNA levels of relevant ABC transporters using RT-PCR. mRNA levels of ABC transporter genes in total liver were quantitated using *18S* as an internal standard. This quantification is complicated by the presence of additional cells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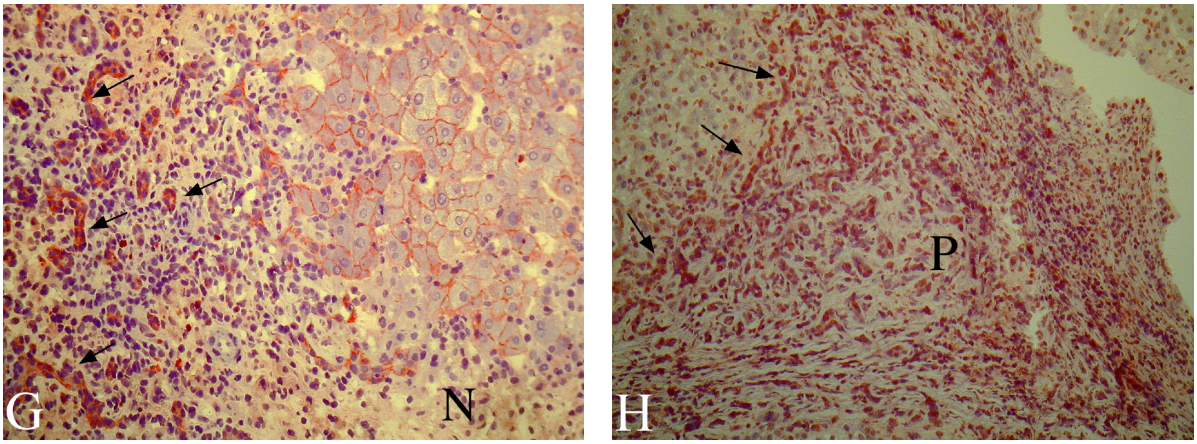

Figure 4.1: Immunohistological staining of MDR1 (A-D), MRP1 (H), and MRP3 (E-G) in human liver biopsies (A) MDR1 staining of normal liver, showing weak immunoreactivity of the parenchymal cells and more intense staining of the interlobular bile duct (arrow)(dilution 1/10). (B) MDR1 expression in severely active HCV, showing very strong apical reactivity of the ductules (arrows) and canalicular staining of the hepatocytes (arrow heads)(dilution 1/50, critical dilution was seen at dilution 1/200). (C) MDR1 expression in submassive necrosis, showing strong immunoreactivity of the regenerating ductules (arrows)(dilution 1/50). (D) MDR1 expression in submassive necrosis, showing increased immunoreactivity of the remaining hepatocytes (arrows)(dilution 1/50). (E) MRP3 expression in normal liver, showing weak immunoreactivity of the pericentral hepatocytes (dilution 1/10). C: central vein. (F) MRP3 expression in PBC, showing basolateral immunoreactivity of the periportal hepatocytes (arrows) and ductules (arrow heads). The interlobular bile ducts (large arrow) stain negative at this dilution, underlining the high expression of MRP3 in the ductules (dilution 1/100). (G) MRP3 expression in submassive necrosis, showing the strong immunoreactivity of the ductules (arrows) and a honeycomb-pattern of hepatocytes (dilution 1/100). (H) MRP1 expression in severely active HCV showing positivity in reactive ductules (arrows) (dilution 1/5). N: necrotic area, P: portal tract

in severely diseased liver, where fibroblasts in fibrotic septa or inflammatory cells may contribute to the *18S* signal. We could not identify better internal standards eliminating this problem. Nonetheless, the RT-PCR data of total liver support the results obtained by immunohistochemistry.

In a previous report on expression of hepatobiliary transporters in human cholestatic liver diseases, no up-regulation of MDR1, or MRP3 was seen.[19] We show in the present study a 5-fold up-regulation of MDR1 and a 10-fold up-regulation of MRP3 in liver specimens of PBC patients. This difference is probably related to the stage of the disease: most of our specimens were in a late stage, since they were mainly pre-transplant hepatectomy specimens. In addition, the dilution of the antibodies until the critical staining level permitted us to evaluate a slight up- or down-regulation. Zollner *et al.*[19] used fluorescence staining methods and only one dilution, which does not permit this evaluation.

We have also studied the ABC transporter gene expression in livers of rats treated with 2-acetylaminofluorene (2-AAF) followed by 70% partial hepatectomy. This activates the progenitor cell compartment. These cells expressed high levels of Mrp1 and Mrp3 (Ros *et al.*, submitted). This correlates with the high expression levels of MRP1 and MRP3

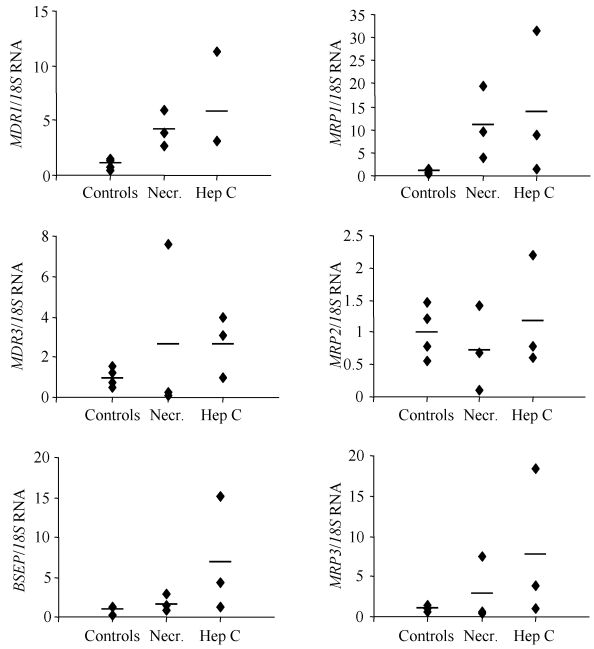

Figure 4.2: mRNA levels of ABC transporter genes in human liver specimens. ABC transporter gene expression in samples from patients with normal liver, submassive necrosis (Necr.) or chronic hepatitis C (Hep C) were determined by real time detection RT-PCR as described in Materials and methods. Values are expressed relative to normal liver, n=3-4 per group.

observed in this study. Unlike the human samples, rat liver progenitor cells in the 2-AAF/PHx model did not express high levels of P-glycoproteins. This difference may be related to the severity of damage in the human liver specimens compared to the rat model. In addition, MDR1 expression levels in human liver are in general higher than Mdr1a/b levels in laboratory animals.

In conclusion, using immunohistochemistry and dilution series, we showed a strong up-regulation of apical MDR1 and basolateral MRP1 and MRP3 in human hepatocytes and in progenitor cell-related bile ductules, during hepatitis or chronic cholestasis. We hypothesize that this reflects a protective mechanism against the accumulation of toxic bile constituents and may render these cells resistant to oxidative stress.

4.6 Acknowledgment

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Chapter 5

Induction of *Mdr1b* expression by tumor necrosis factor-α in rat liver cells is independent of p53 but requires NF-κB signaling

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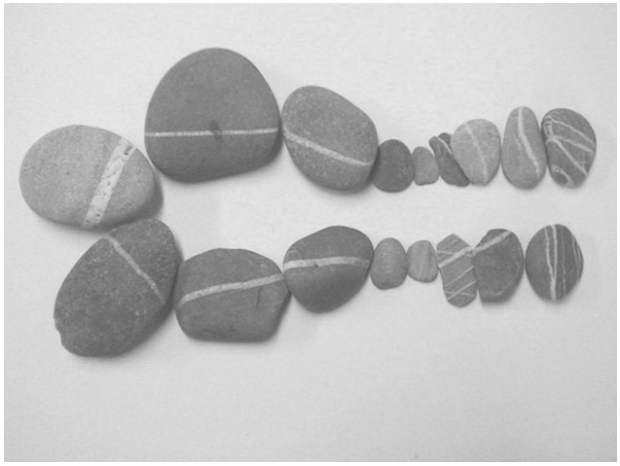

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adapted from: Hepatology 2001; 33:1425-1431

5.1 Abstract

The multidrug resistance protein *Mdr1b* in rats is up-regulated during liver regeneration after partial hepatectomy or after endotoxin treatment. We hypothesize that up-regulation of *Mdr1b* in these models is TNF-α-dependent. The mechanism of *Mdr1b* activation by TNF-α is unknown as TNF-α can signal through various pathways, including NF-κB and p53, transcription factors for which binding sites in the *Mdr1b* promoter have been identified. We aimed to elucidate the mechanism of up-regulation of *Mdr1b* by TNF-α. We selectively used constructs expressing dominant negative Fas-associated death domain protein (FADD), TNF receptor associated factor-2 (TRAF2) or IκB to inhibit pathways downstream of the TNF receptor. Further, the proteasome inhibitor MG-132 was used which prevents the breakdown of IκB. We show a critical role for NF-κB in activation of *Mdr1b* gene expression both in primary rat hepatocytes and in rat hepatoma H-4-II-E cells. Because p53 is up-regulated by TNF-α in an NF-κB-dependent manner and the *Mdr1b* promoter contains a p53 binding site, we used liver cells expressing a dominant negative p53 to show that TNF-α up-regulation of *Mdr1b* is independent of functional p53. Using transient transfection assays we show that *Mdr1b* up-regulation correlates with activation of the promoter. Mutation of the NF-κB site in the *Mdr1b* promoter prevents its induction by TNF-α. In conclusion our results show that activation of the rat *Mdr1b* gene by TNF-α is a result of NF-κB signaling and independent of p53.

5.2 Introduction

The human MDR1 (ABCB1) and its rodent homologues Mdr1a and Mdr1b belong to subcluster B of the ATP-binding cassette transporter superfamily. These transporters can confer multidrug resistance (MDR) to cells by functioning as efflux pumps for cytotoxic drugs (for review see Klein *et al.*[1]). MDR1 and Mdr1a/1b are expressed in the blood-brain barrier, small intestine, kidney, and, to a lesser extent, in the liver where they presumably have an important role in the elimination of endogenous and exogenous compounds.

Whereas the expression of hepatic *Mdr1a* is largely unchanged, rat *Mdr1b* is up-regulated under various conditions and appears to be a "stress-responsive" gene. In cell culture, *Mdr1b* expression is influenced by the extracellular matrix.[2,3] In addition, *Mdr1b* expression increases in response to, for example, cytotoxic drugs,[4–6] carcinogens,[7–9] insulin,[10] or hydrogen peroxide.[11] *In vivo*, *Mdr1b* expression is increased in hepatocarcinogenesis, during liver regeneration, and endotoxin-induced cholestasis.[12–14] The cytokine tumor necrosis factor-alpha (TNF-α) plays an essential role both in liver regeneration after partial hepatectomy[15,16] and in lipopolysaccharide-induced endotoxemia.[17] We therefore speculate that TNF-α is, at least in part, responsible for the up-regulation of *Mdr1b* observed in these models. It has been reported that *Mdr1b* expression can be induced by TNF-α in cultured rat hepatocytes,[18] but the underlying mechanisms have not been elucidated.

TNF-α induces a well-described signaling cascade leading to caspase activation, but also leads to activation of NF-κB and MAP kinases. Activation of caspases involves binding of TNF-receptor-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD) to the TNFR1 receptor. Caspase activation results in apoptosis when anti-apoptotic pathways are not effective. Activation of MAP kinases and NF-κB by TNF-α involves a cascade of factors, starting with TRADD and TNF-receptor associated factor-2 (TRAF2)/receptor-interacting protein (RIP). NF-κB activation subsequently requires activation of NF-κB-inducing kinase (NIK), and IκB kinases (IKK). Alternative pathways for the activation of IκB kinases have been shown. Reactive oxygen species, that are induced by TNF-α,[19,20] can directly act on the IκB kinases.[9,21] NF-κB is retained in the cytoplasm by IκB. For activation of NF-κB, phosphorylation of IκB by IκB kinases is essential, followed by its ubiquitination and degradation by the 26S proteasome. This releases the NF-κB subunits, which then translocate to the nucleus (for review see Wallach *et al.*,[22] and Ashkenazi and Dixit,[23]). Thus, inhibition of NF-κB signaling can occur by either blocking proteasome mediated degradation with chemical proteasome inhibitors or using mutant IκBs that cannot be phosphorylated.[24]

Oxidative stress, as induced by TNF-α, results in activation of p53.[25] Various cellular mechanisms have been found to activate p53, including NF-κB.[26] The *Mdr1b* promoter contains a p53-binding site, and therefore *Mdr1b* induction by TNF-α can involve p53.[4] Moreover, NF-κB and p53 have been demonstrated to functionally interfere with each others transcriptional acti vation.[27,28] It is unknown how these two pathways interact in the regulation of *Mdr1b* by TNF-α because the *Mdr1b* promoter contains an NF-κB binding site in proximity to a p53 binding site.[4,10]

The aim of the present study was to elucidate the intracellular mechanisms of TNF-α-induced *Mdr1b* expression in liver cells. We used *in vitro* cultures of either primary rat hepatocytes or rat hepatoma cells and modulated the TNF-α-induced pathways with specific inhibitors. The role of p53 in the up-regulation of *Mdr1b* by TNF-α was evaluated using rat hepatoma cells expressing dominant negative p53.

5.3 Materials and methods

Materials

Cell culture media, fetal calf serum (FCS) and oligonucleotides were from Life Technologies Ltd. (Paisley, UK). Antibiotics came from BioWhittaker (Walkersville, MD). Recombinant mouse TNF-α was purchased from R&D Systems (Abingdon, UK).

Cells and culture conditions

Rat hepatoma H-4-II-E cells (European Collection of Cell Culture, Salisbury, UK) were maintained in Earles modified Eagles medium supplemented with 10% FCS, 2 mmol/L glutamine, non-essential amino acids and penicillin/streptomycin/fungizone. The rat hepatoma cell lines H35 TDN7 and NEO17 were maintained as described previously.[29] For experiments, cells were seeded on rat tail collagen gels at a density of 2×10^5 cells/cm^2.

Primary rat hepatocytes were isolated from male Wistar rats using a two-step collagenase perfusion as described elsewhere.[30] Cells were seeded in Williams medium E supplemented with 10% FCS, 50 nmol/L dexamethasone (Sigma, St Louis, MO), 20 mU/mL insulin (Novo Nordisk, Bagsvaerd, Denmark) and penicillin/streptomycin/fungizone at a density of 1.5×10^5 cells/cm^2.

The complete medium was replaced by serum-free medium (cell lines) or serum- and dexamethasone-free medium (primary rat hepatocytes) 4 hours after seeding. Cells receiving adenovirus were then infected for 12 hours, followed by a 12-hour recovery period. TNF-α incubations were started 28 hours after seeding. The proteasome inhibitor MG-132 (Calbiochem, La Jolla, CA) was added at a final concentration of 84 nmol/L 30 minutes before addition of TNF-α where indicated. All cells were maintained in a humidified incubator at 37°C/5% CO_2.

Adenovirus constructs

The adenovirus constructs used have been described before.[31–33] The IκB super-repressor adenovirus Ad5IκBAA contains an IκB construct in which serines 35 and 36 have been replaced by alanines. This mutated IκB cannot be phosphorylated and binds NF-κB irreversibly, preventing translocation of NF-κB to the nucleus.[31] Ad5dnFADD expresses a FADD mutant lacking the death effector domain, that is therefore unable to bind caspase 8.[32] Ad5dnTRAF2 contains a TRAF2 in which the N-terminal region of the ring finger domain has been deleted, preventing interaction with downstream signaling molecules.[33] Ad5β-Gal, expressing the *Escherichia coli* β-galactosidase gene, was used as a control virus throughout the experiments.[31] Cells were infected at an moi of 10 (primary hepatocytes) and 50 (H-4-II-E cells) as determined by plaque assay. For co-infections, cells were infected

with Ad5IκBAA and Ad5dnFADD at an moi of 10 (primary hepatocytes) and 50 (H-4-II-E).

RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

Prior to RNA isolation, cells were incubated with 300 μg/mL collagenase (Sigma) for 15 minutes. RNA was isolated using the SV Total RNA isolation system (Promega, Madison, WI) according to manufacturer's instructions. Reverse transcription was performed on 5 μg of total RNA using random primers in a final volume of 75 μL (Reverse Transcription System, Promega). Three microliters of cDNA was used in each PCR reaction in a final volume of 50 μL, containing 50 pmol of sense and of antisense primers, 0.2 mmol/L dNTPs (Pharmacia, Uppsala, Sweden) and 0.5 U EuroTaq Polymerase (Eurogentech, Seraing, Belgium). Primers used were 5'-CTA TTG CGC CGC TAG AGG TG-3' (sense) and 5'-CTG AAC GCC ACT TGT CCC TC-3' (antisense) for *18S* (product size 525 base pairs), 5'-ATG TTC CGA GAG CTG AAT GAG G-3' (sense) and 5'-GGA CTA GCA TTG TCT TGT CAG C-3' (antisense) for *p53* (product size 270 base pairs) and 5'-GAA ATA ATG CTT ATG AAT CCC AAA G-3' (sense) and 5'-GGT TTC ATG GTC GTC GTC TCT TGA-3' (antisense) for *Mdr1b* (product size 325 base pairs). For each primer set an increasing number of PCR cycles was performed to ensure that all data were obtained from samples in the exponentional phase. The number of cycles used was 15-17 for *18S*, 25 for *p53* and 28 for *Mdr1b*. PCR reactions were performed as described before.[13] *18S* and *Mdr1b* RNA levels were related to internal competitor fragments made according to Celi *et al.*[34] These fragments were generated using the sense primers as mentioned above and the antisense primers 5'-CTG AAC GCC ACT TGT CCC TCA GAC AAA TCG CTG CAC CAA C-3' and 5'-GGT TTC ATG GTC GTC GTC TCT TGA GCA CCC ATT TAT AAC AGC ACA AA-3' for *18S* and *Mdr1b*, respectively. Ten microliters of each PCR product were loaded on a 2.5% agarose gel and stained with ethidium bromide. Band intensities of digitized images were quantified using Imagemaster 1D Elite software version 3.00 (Pharmacia).

Nuclear extracts

Nuclear extracts were prepared from H-4-II-E cells as described previously.[35] Cells were washed twice with PBS, harvested in 500 μL PBS, spun down and resuspended in 400 μL of buffer containing 10 mmol/L Hepes, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT and Complete (Roche Diagnostics, Almere, the Netherlands). After a 15-minute incubation on ice, Nonidet-P-40 was added to a final concentration of 0.25%. After centrifugation, the pellet was resuspended in 50 μL of buffer containing 20 mmol/L Hepes, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT and Complete. After constant agitation for 30 minutes at 4°C, nuclear debris was pelleted by centrifugation. The supernatant was stored at -80°C until analysis.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed with approximately 5 μg of nuclear proteins in a total volume of 15 μL in a buffer containing 20 mmol/L Hepes pH7.9, 60 mmol/L KCl, 0.06 mmol/L

EDTA, 0.6 mmol/L DTT, 2 mmol/L spermidine, 10% glycerol, 2 μg poly [d(I-C)] (Roche Diagnostics) and radiolabeled probe at 26°C for 25 min.[35] DNA probe was radiolabeled with [α-^{32}P]dATP (Amersham, Buckinghamshire, UK) using Klenow Polymerase (Promega). The mdr-κB probe used, 5'-gat cCT GGG GAA TTC CAG CTC-3' with the NF-κB site underlined, has been published before.[10] The consensus sequence for AP-1 was used in competition experiments. For supershifts, samples were incubated with a p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 30 minutes before the addition of the radiolabeled probe. The final reaction mixture was analyzed on a 4% polyacrylamide gel with 0.5 × Tris borate-EDTA electrophoresis buffer.

Plasmids

The rat *Mdr1b*-luciferase reporter construct -736WT-Luc has been described.[7] The mdr-κB mutant -736κM-Luc was generated by direct site directed mutagenesis (QuikChange, Stratagene, LaJolla, CA) using -736WT-Luc as a template and 5'-TCT GTG TTA ATG TCT GCT CAA TTC CAG CTC CCT T-3' and its complementary sequence as primers (mutated base pairs are underlined), analogous to Zhou and Kuo.[10]

Transient transfections and luciferase assays

10^5 H-4-II-E cells, cultured on plastic, were transfected using Effectene (Qiagen, Hilden, Germany) with 0.6 μg of Qiagen Endofree purified plasmid DNA according to manufacturers instructions. After 12 hours, medium was replaced by serum-free medium. Cells were lysed 30 hours later by incubation with passive lysis buffer (Promega). Luciferase activity was assayed on 20 μL of lysate using the luciferase assay system (Promega), utilizing the Anthos LUCY1 luminometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) with a 10-s counting window. Luciferase activity was normalized to the amount of living cells as determined by a standard microculture tetrazolium test (MTT). We were unable to normalize to β-galactosidase or *Renilla* luciferase as TNF-α had a strong effect on the promoters driving the expression of these reporters.

5.4 Results

TNF-α induces Mdr1b expression in rat hepatoma cells and in primary rat hepatocytes

Rat hepatoma H-4-II-E cells cultured on collagen gels and serum-starved for 24 hours were treated with TNF-α (20 ng/mL) for different time intervals. As shown in Figure 5.1, (left), *Mdr1b* mRNA levels increased within 3 hours of TNF-α addition and reached a maximal level of approximately 4.5-fold after 6 to 9 hours (Figure 5.1, right). Likewise, when primary rat hepatocytes cultured under comparable conditions were treated with TNF-α, an approximately 3-fold increase in *Mdr1b* mRNA levels was observed after 12 hours (data not shown).

IκBAA inhibits induction of nuclear NF-κB binding activity by TNF-α

We selectively inhibited the various pathways activated by TNF-α using adenoviral dominant expression constructs for mutated TRAF2, FADD or IκB with an adenovirus

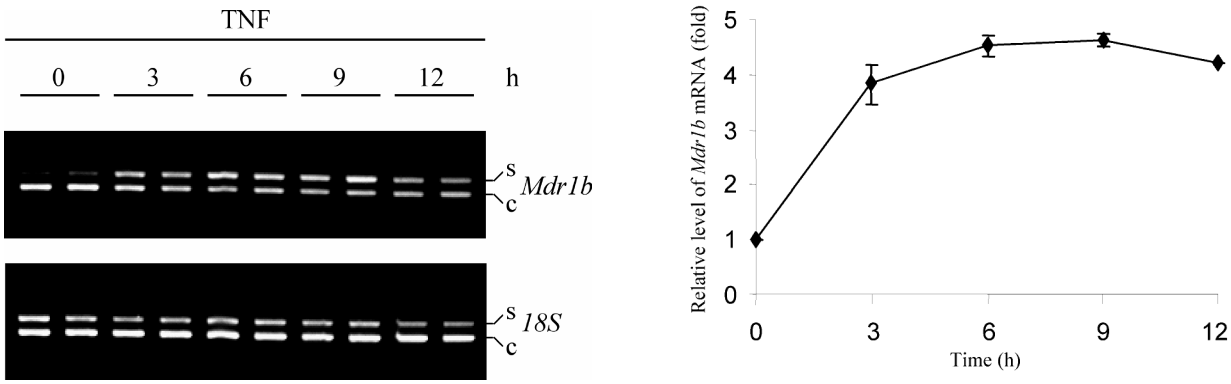

Figure 5.1: TNF-α-induced expression of the rat *Mdr1b* gene. Left panel: competitive RT-PCR analyses for *Mdr1b* and *18S* in H-4-II-E cells. s, signal from sample; c, signal from the competitor fragment. Cells were cultured on collagen and serum-starved for 24 hours prior to incubation with TNF-α (20ng/mL) for different times as indicated. RNA was isolated and RT-PCR performed as described in the Materials and methods. Data are representative of results from 2 independent experiments performed in duplicate. Right panel: quantitative results of time-dependent induction of rat *Mdr1b* mRNA by TNF-α. Data points represent means ± S.D. from 2 independent experiments performed in duplicate.

expressing β-galactosidase as a control. Functionality of the different viral constructs was shown in multiple ways. Ad5IκBAA inhibits NF-κB activation as shown by EMSA (Figure 5.2). Ad5dnFADD inhibits apoptosis. Whereas cells exposed to TNF-α following treatment with IκBAA die within 4 to 5 hours, combined treatment with dnFADD prevents this. Moreover, caspase-3 activation as observed after treatment of cells with TNF-α in combination with actinomycin-D is inhibited by pretreatment with dnFADD (data not shown). Expression of exogenous TRAF-2 was determined by RT-PCR. Functionality was shown by Western blot analysis using an antibody raised against phospho-c-Jun. Exposure of control cells to TNF-α resulted in an increase in phospho-c-Jun in nuclear cell extracts, which was absent in cells pretreated with dnTRAF2 (data not shown).

To study the effect of TNF-α and the various adenoviral constructs on the nuclear NF-κB binding activity, we performed EMSAs using the NF-κB binding site of the rat *Mdr1b* promoter (mdr-κB) as a probe. Nuclear extracts were prepared from H-4-II-E cells in the presence or absence of TNF-α. As shown in Figure 5.2, (left), stimulation with TNF-α resulted in a clear formation of a DNA-protein complex. This complex was absent in cells expressing the IκB super-repressor (lanes 17-20). The DNA-protein complex was competed by unlabeled mdr-κB (Figure 5.2 [right], lanes 2 and 3) but not by an excess of unlabeled unrelated probe (AP-1, lanes 4 and 5). Supershift analysis with NF-κB p65 antibody showed protein specificity of binding to the mdr-κB oligonucleotide (lane 6).

Mdr1b expression induced by TNF-α is dependent on activation of NF-κB

To investigate the intracellular signal transduction pathway involved in the induction of rat *Mdr1b* by TNF-α, we selectively inhibited the pathways with the various adenoviral constructs. When the NF-κB pathway was inhibited with IκBAA, incubation of the cells

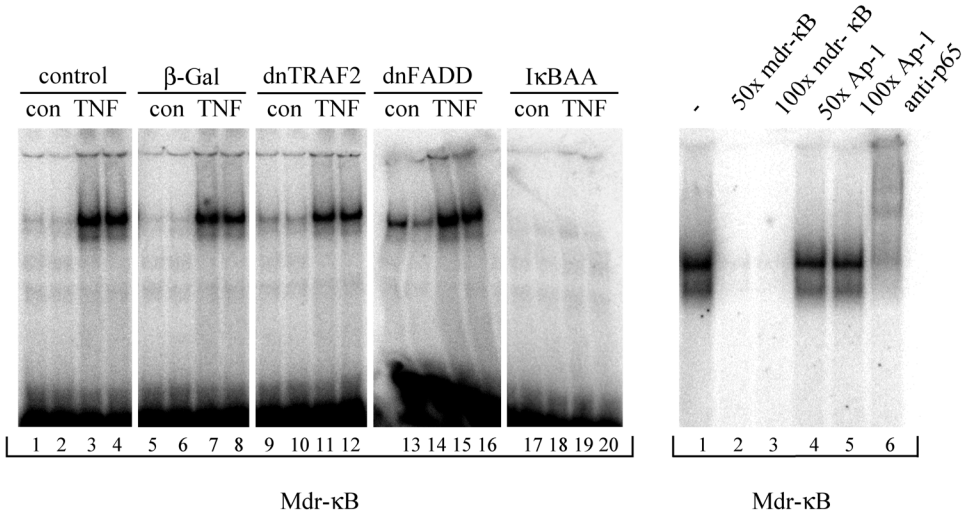

Figure 5.2: Activation of nuclear NF-κB activity in H-4-II-E cells by TNF-α. Left panel: H-4-II-E cells were cultured on collagen and infected with the various adenovirus constructs as indicated. Twenty-four hours after serum removal and 12 hours after virus removal cells were treated with TNF-α (20 ng/mL) for 2 hours. Nuclear extracts were prepared and assayed for NF-κB binding activity as described in Materials and methods. con, control. Right panel: nuclear extracts from uninfected TNF-α-treated cells were assayed for NF-κB binding activity in the absence or presence of excess unlabeled competitors (mdr-κB, AP-1) or NF-κB p65 antibody as indicated.

with TNF-α for periods longer than four hours resulted in massive cell death. Therefore, in these experiments IκBAA was used in combination with dnFADD to simultaneously inhibit the activation of both NF-κB and caspases. Twelve hours after the removal of the virus-containing media, the cells were incubated with TNF-α for 9 hours. Figure 5.3 (top) shows that in H-4-II-E cells induction of *Mdr1b* expression by TNF-α is observed under all conditions except when the cells were treated with IκBAA/dnFADD, indicating that NF-κB plays a crucial role in the induction of *Mdr1b* by TNF-α. When primary rat hepatocytes were treated either with IκBAA/dnFADD or with the proteasome inhibitor MG-132, the expression of *Mdr1b* was decreased both in cells stimulated with TNF-α and in untreated cells (Figure 5.3, bottom). This indicates that in cultured primary rat hepatocytes, NF-κB is important not only for the induction of *Mdr1b* by TNF-α but also for the basal expression of *Mdr1b*.

TNF-α induces p53 via the NF-κB pathway

It has been reported that NF-κB activates the p53 promoter.[26] We therefore tested whether TNF-α increased endogenous p53 mRNA levels in H-4-II-E cells. Figure 5.4 (top) shows that p53 mRNA expression was induced time-dependently by TNF-α and reached a maximum after 6 to 9 hours. We compared the expression of p53 in H-4-II-E cells with

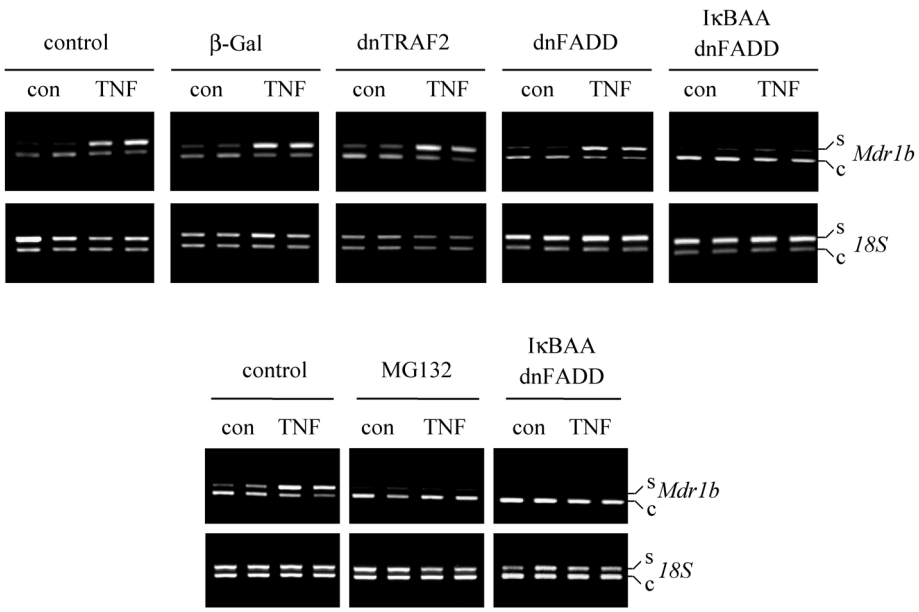

Figure 5.3: TNF-α-induced expression of the rat *Mdr1b* gene is NF-κB dependent. Competitive RT-PCR analyses for *Mdr1b* and *18S* in H-4-II-E cells (upper panel) and primary rat hepatocytes (lower panel). Cells were cultured on collagen and serum-starved for 24 hours prior to incubation with TNF-α (20 ng/mL) for 9 hours. Cells were infected with adenoviral constructs or treated with MG-132 as indicated. RNA was isolated and RT-PCR performed as described in Materials and methods. s, signal from sample; c, signal from the competitor fragment. Data are representative of results from 2 independent experiments performed in duplicate.

that in H-4-II-E cells transduced with dnTRAF2, dnFADD or IκBAA in combination with dnFADD to see which pathway activated by TNF-α is involved in this induction. Figure 5.4 (bottom) shows that induction of p53 mRNA expression by TNF-α is abolished in cells where the NF-κB pathway is inhibited by IκBAA/dnFADD transduction.

Mdr1b expression induced by TNF-α is not dependent on p53 activation

As the *Mdr1b* promoter contains a p53 binding site and TNF-α induces p53 via NF-κB, we next investigated the possible involvement of p53 in the induction of rat *Mdr1b* gene expression by TNF-α. We compared the effect of TNF-α treatment on *Mdr1b* mRNA levels in three different cell lines: H-4-II-E, H35 TDN7 and H35 NEO17. H35 TDN7 cells are stably transfected with trans-dominant negative (TDN) p53. Presence of the dominant negative p53 construct was confirmed by determination of p53 levels by Western blot analysis and *Mdr1a* mRNA levels by RT-PCR. The results (data not shown) conformed with those from Thottassery *et al.*,[29] who showed that loss of functional p53 results in increased expression of *Mdr1a*. H35 NEO17 cells contain the backbone vector CMV-Neo-Bam and were used as a control.[29] These cells, cultured on collagen and serum-starved for 24 hours, were treated with TNF-α (20 ng/mL) for 12 hours before RNA isolation. As shown in Figure 5.5, (left), *Mdr1b* mRNA levels increased similarly in the 3 different cell

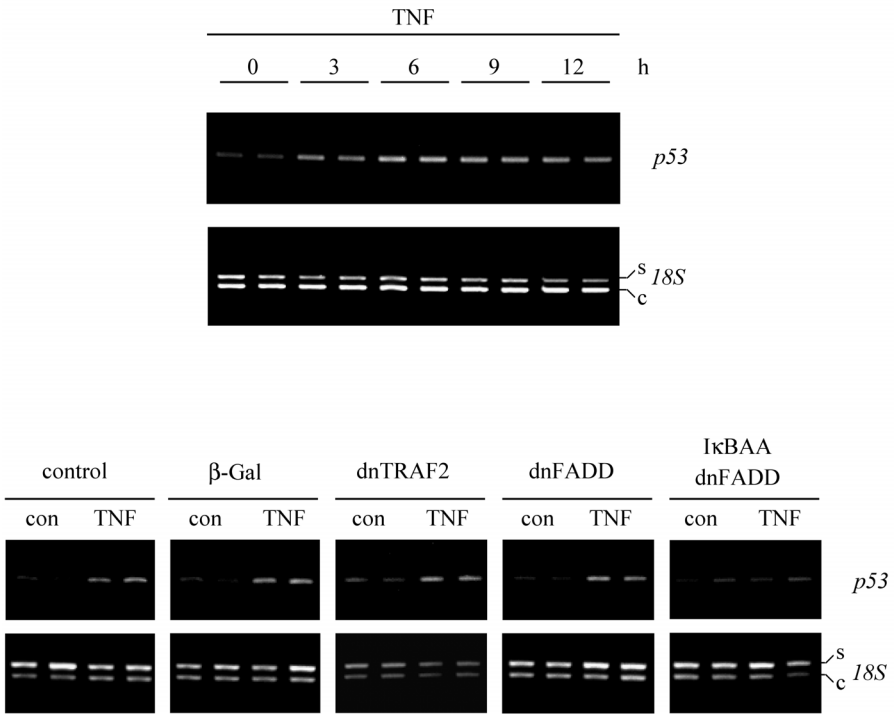

Figure 5.4: Increase in *p53* mRNA level in H-4-II-E cells by TNF-α is NF-κB dependent. RT-PCR analyses for *p53* and *18S* in H-4-II-E cells. Cells were cultured on collagen and serum-starved for 24 hours before incubation with TNF-α (20 ng/mL) for different times as indicated. RNA was isolated and RT-PCR performed as described in Materials and methods. For *18S* PCR: s, signal from sample; c, signal from the competitor fragment. Data are representative of results from 2 independent experiments performed in duplicate. Upper panel: time course of induction of rat *p53* mRNA by TNF-α. Lower panel: rat *p53* mRNA levels after incubation with TNF-α for 9 hours. Cells were infected with adenovirus constructs as indicated.

lines. Increases of 4 to 5-fold were observed in this experiment (Figure 5.5, right). These results show that the induction of rat *Mdr1b* expression by TNF-α is independent of p53 because cells lacking functional p53 up-regulate *Mdr1b* just as readily as those that retain functional wild-type p53.

TNF-α induces the rat Mdr1b promoter through its NF-κB binding site

To investigate whether binding of NF-κB to the rat *Mdr1b* promoter was sufficient for the induction of *Mdr1b* by TNF-α, we performed transient transfection assays using a rat *Mdr1b* promoter construct coupled to the luciferase gene. When -736WT-Luc, containing 736 base pairs of the rat *Mdr1b* promoter plus 608 base pairs downstream from the transcription start site, was transfected into H-4-II-E cells followed by TNF-α treatment, luciferase activity increased approximately 2.8-fold compared to controls (Figure 5.6). This induction is lower than found for endogenous *Mdr1b* (Figure 5.1). However, in transfection experiments, cells could not be cultured on collagen as this would interact with the DNA-liposome particles. Moreover, the transfection itself can induce stress. This could result

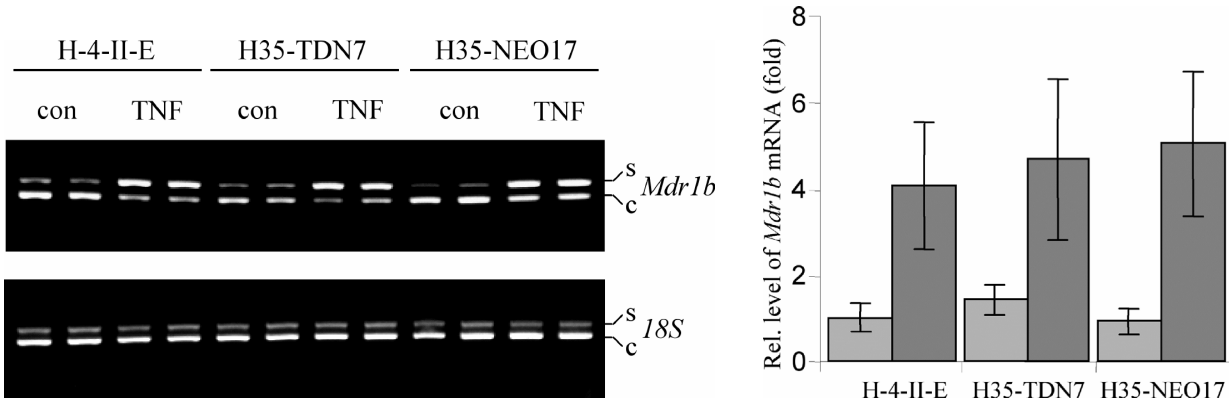

Figure 5.5: Trans-dominant negative p53 expression in H35 hepatoma cells does not affect the induction of *Mdr1b* gene expression by TNF-α. Left panel: competitive RT-PCR analyses for *Mdr1b* and *18S* in H-4-II-E, H35 TDN7 and H35 NEO17 cells. Cells were cultured on collagen and serum-starved for 24 hours before treatment with TNF-α for 12 hours. RNA was isolated and RT-PCR performed as described in Materials and methods. s, signal from sample; c, signal from the competitor fragment. Right panel: quantitative results of TNF-α-induced expression of the rat *Mdr1b* gene in H-4-II-E, H35 TDN7 and H35 NEO17 cells. Values are normalized to control values for H-4-II-E cells. Data points represent means ± S.D., n=2. light gray = -TNF-α, dark gray = +TNF-α.

in increased basal expression levels of the *Mdr1b*-reporter construct, and consequently a lower fold of induction. Mutation of the mdr-κB site abolished this activation completely and instead caused a repression of the *Mdr1b* promoter. These results demonstrate that a functional mdr-κB site is required in the rat *Mdr1b* promoter to allow induction of *Mdr1b* by TNF-α.

5.5 Discussion

In this study we show a time-dependent stimulation of the rat *Mdr1b* expression by TNF-α in H-4-II-E rat hepatoma cells as well as in primary rat hepatocyte cultures. We show that NF-κB activation is essential for TNF-α-induced *Mdr1b* expression. Moreover, EMSA experiments show that up-regulation of the *Mdr1b* gene correlates with nuclear translocation and binding of NF-κB to its target site in the *Mdr1b* promoter. Although the promoter is up-regulated by TNF-α and this is lost when the mdr-κB site is mutated, we cannot rule out other factors, regulated by NF-κB, playing a role in *Mdr1b* up-regulation. Nevertheless, despite NF-κB increasing p53 (Figure 5.4 and Wu and Lozano[26]), p53 does not play a role in TNF-α up-regulation of *Mdr1b* because a liver cell line expressing a dominant negative p53[29] is still capable of up-regulating *Mdr1b* after TNF-α treatment (Figure 5.5).

On stimulation with TNF-α *Mdr1b* mRNA levels reached a maximum after 6 to 9 hours. A similar time-frame of induction has been observed *in vivo* after endotoxin treatment or partial hepatectomy in rats.[13,14] Induction of *Mdr1b* mRNA by TNF-α has been demonstrated previously by Hirsch-Ernst *et al.*, in primary rat hepatocyte cultures, yet, in their

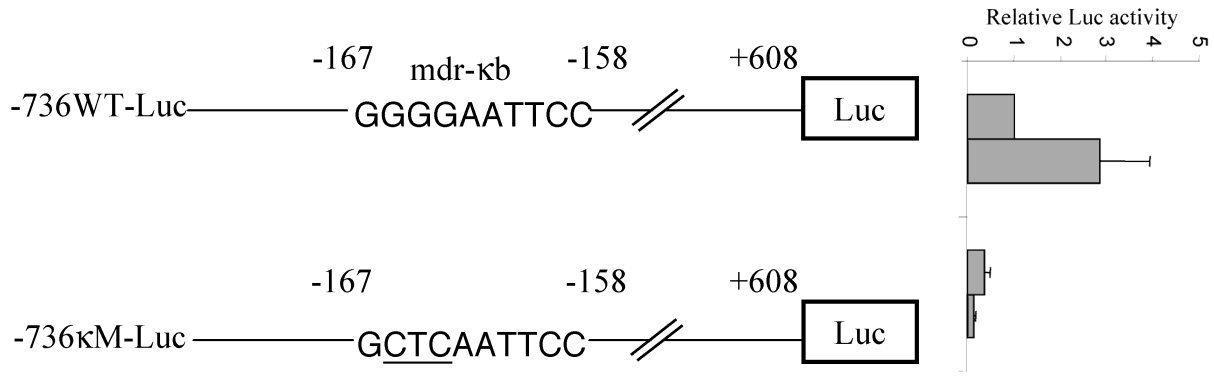

Figure 5.6: A functional mdr-κB site is required for TNF-α-induced *Mdr1b* promoter activity. H-4-II-E cells were transfected with the rat *Mdr1b* promoter constructs as described in Materials and methods. Cells were stimulated with TNF-α (20 ng/mL) for 18 hours before lysis. Values are expressed relative to control values for the wild-type construct. Data points represent means from 3 independent experiments. light gray = -TNF-α, dark gray = +TNF-α. S.D. values are represented by bars.

system, maximal *Mdr1b* mRNA levels were reached only after 3 days of incubation with TNF-α.[18] In their experiments, TNF-α was added 4 hours after seeding the cells whereas in our system TNF-α was added to the cells 28 hours after plating. This and other differences in culture conditions might explain the divergent time kinetics observed.

Besides NF-κB and p53 binding sites, other cis-elements have been identified in the rat *Mdr1b* promoter, including Sp1 and AP-1 binding sites.[7,36] The Sp1 binding site is essential for optimal basal activity.[7] Our findings with both MG-132 and IκBAA leading to decreased levels of *Mdr1b* in primary rat hepatocyte cultures support the idea that NF-κB also plays a role in regulating basal *Mdr1b* expression.[10] It is unlikely that Sp1 plays a role in NF-κB-mediated regulation of the *Mdr1b* promoter because the *Mdr1b* promoter with the mutated NF-κB binding site still retains these elements and is not activated by TNF-α. Further, as deletion of the AP1 site has no effect on the promoter activity, AP-1 does not appear to play a role in its regulation.[7]

TNF-α induces both anti-apoptotic and pro-apoptotic pathways. Activation of the NF-κB pathway results in activation of genes that mediate protective functions because after inhibition of NF-κB activation, the apoptotic pathways prevail.[37] This also occurred in our system when cells treated with the IκB super-repressor were exposed to TNF-α for periods longer than 4 hours. It is possible that up-regulation of *Mdr1b* may help to protect the cell against apoptosis. A direct relation between Mdr1 over-expression and apoptosis was established by Johnstone *et al.*,[38] who observed that a T-cell leukemia cell line transduced with a retroviral construct containing human MDR1 was more resistant to TNF-α-induced apoptosis than control cells. Also drug-selected cell lines have been shown to be less sensitive to apoptosis, but the cytotoxic compounds used to select resistant cells may induce other changes besides up-regulation of Mdr1 proteins.[39–41] In these studies PSC833 is often used as an Mdr1 inhibitor, but may itself increase apoptosis.[42]

Regarding the mechanism involved, it has been observed that increased expression of MDR protein lowers the sensitivity of the cell to caspase-dependent apoptosis by decreasing the caspase-3 activity.[40] Because MDR proteins can transport small peptides,[43] it is possible that MDR proteins modulate the levels of proteins essential for effective activation of caspase-3. Alternatively, it has been proposed that MDR proteins affect the intracellular pH.[38] Thirdly, MDR proteins may influence intracellular ceramide levels.[39] TNF-α treatment increases intracellular ceramide levels.[44] Primary rat hepatocytes are, however, resistant to ceramide treatment unless RNA synthesis is inhibited,[45] implying that transcriptional up-regulation of protective factors is required. Up-regulation of MDR1 has been reported to increase the translocation of sphingomyelin from the inner to the outer leaflet of the cell membrane, thereby keeping intracellular concentrations of sphingomyelin low and preventing its conversion to ceramide by neutral sphingomyelinases.[39]

An alternative explanation for the profound up-regulation of *Mdr1b* expression during acute phase response is that its expression is intimately coupled to the expression of cytochrome P450 genes. During liver regeneration after partial hepatectomy, endotoxin treatment, or bile duct ligation the levels of various cytochrome P450 proteins are decreased whereas *Mdr1b* is up-regulated.[46–48] TNF-α can also down-regulate cytochrome P450 proteins, including P450 2C11.[49] Schuetz *et al.* have recently demonstrated the interrelationship between the expression of cytochrome P450 and *Mdr1a/1b* levels. Members of the cytochrome P450 family were up-regulated in *Mdr1a*, *Mdr1a/b*, and to a lesser extent in *Mdr1b* nullizygous mice.[50] Up-regulation of *Mdr1b* by the cell could be a compensatory pathway to enable transport of compounds no longer metabolized via cytochrome P450 directly into the bile.

The rapid up-regulation of *Mdr1b in vivo* after partial hepatecomy and during endotoxin-induced cholestasis implies that *Mdr1b* is part of the hepatic acute phase response.[13,14] This article shows that expression of *Mdr1b* by TNF-α is under NF-κB control. Clearly future studies will elucidate how *Mdr1b* affects apoptotic signaling and impacts cell survival.

5.6 Acknowledgment

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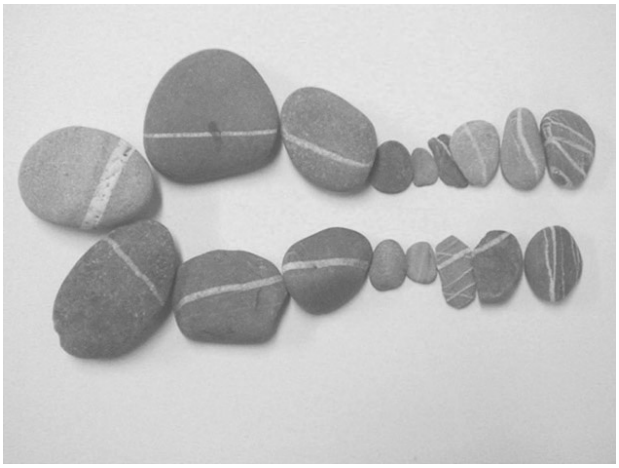
Summary and general discussion



Important physiological functions of the liver include the metabolism and disposal of (potentially) toxic endo- and xenobiotics. These compounds are secreted by the liver for removal via bile or urine. This secretion involves specific transport proteins that, in most cases, belong to the ATP-binding cassette (ABC) transporter superfamily. ABC transporters have the capacity to actively transport compounds out of the cell against steep concentration gradients. In this way, liver cells lower the intracellular concentrations of potentially toxic compounds. High expression levels of specific ABC transporters can thus protect the cell from damage.

The liver has the capacity to regenerate after loss of tissue. A commonly used model to study liver regeneration in experimental animals is the surgical removal of ∼70% of the liver (partial hepatectomy, PHx). Under these circumstances, the remaining liver cells will start to replicate but at the same time must maintain their normal metabolic functions as good as possible. In **chapter 2** the expression of hepatic transport systems involved in bile formation during liver regeneration after PHx in rats has been studied. Initial studies showed maximal DNA synthesis at 24 hours after PHx. Therefore, transporter expression and bile formation were analyzed in detail at this timepoint. Serum bile salt levels were highly increased after PHx, as a result of the reduced hepatic transport capacity. The expression of hepatic uptake transporters was markedly decreased, limiting the uptake of bile salts into the hepatocyte. The expression of proteins involved in bile salt secretion was, however, maintained at a normal level. As bile flow and bile salt secretion were increased when expressed per gram liver, the remnant liver was not cholestatic. Hepatocytes in the remnant liver showed highly increased levels of *Mdr1b* mRNA, which may increase the resistance of hepatocytes against products of cellular oxidative stress reactions, such as lipid peroxidation. In addition, a number of cytochrome P450 proteins are known to be down-regulated after PHx, possibly to reduce formation of reactive oxygen species and risk of DNA damage during cell division. Up-regulation of *Mdr1b* by the cell could be a compensatory pathway to enable transport of substrates, not metabolized via cytochrome P450, directly into the bile.

Liver damage results in activation of hepatic progenitor cells when proliferation of hepatocytes is suppressed. These progenitor cells are located in the canals of Hering, which form the connection between the hepatocytic canaliculi and the bile ductules. Activation of these progenitor cells results in the generation of bipotential oval cells, able to differentiate into hepatocytes or into cholangiocytes. As these oval cells provide a mechanism for the liver to regenerate after severe injury, they must be well protected against toxic damage. High expression levels of specific ABC transporters would be beneficial in this respect. However, ABC transporter gene expression had thus far not been characterized in oval cells. In rats, oval cell proliferation can be achieved by treatment with 2-acetylaminofluorene (2-AAF), which inhibits proliferation of hepatocytes, after which regeneration is induced by PHx. **Chapter 3** describes the expression profile of ABC transporters in 2-AAF/PHx-treated rats. In total liver the mRNA levels of *Mdr1b* were highly increased. In addition, mRNA levels of *Mrp1* and *Mrp3* were increased. Using immunohistochemistry, we could localize the increased Mdr1b expression to the periportal hepatocytes whereas oval cells expressed Mrp1 and Mrp3.

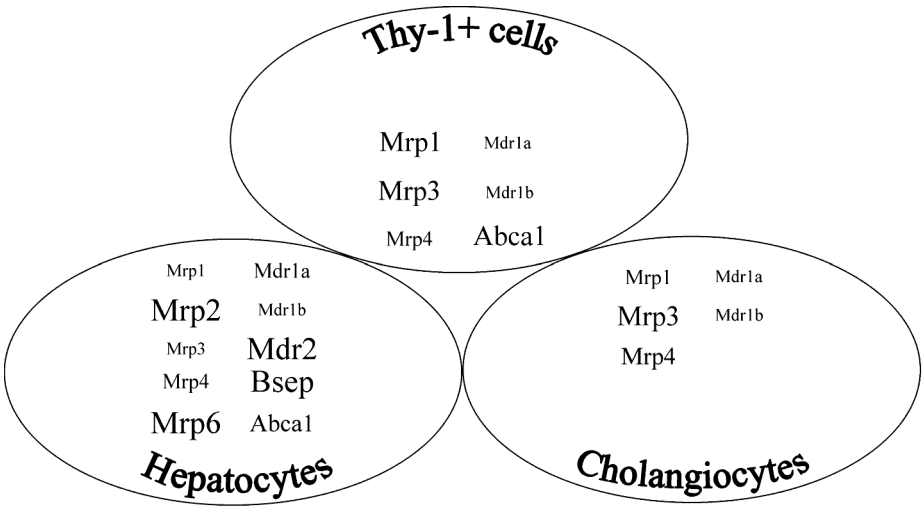


Figure 1: Schematic representation of relative ABC transporter gene expression in hepatocytes, cholangiocytes and Thy-1 positive cells

The oval cells were further characterized by comparing isolated Thy-1 positive cells with isolated cholangiocytes and hepatocytes. It has been shown that Thy-1 expression in the liver is specific for oval cells. This allows the specific isolation of these cells by flow cytometry. Thy-1 positive cells highly expressed *Mrp1* and *Mrp3* mRNA, while the hepatocyte-specific transporters *Mdr2*, *Bsep*, *Mrp2*, and *Mrp6* were minimally expressed. The expression pattern of ABC transporters in Thy-1 positive cells resembled that of cholangiocytes except for *Abca1*, which was expressed in Thy-1 positive cells but not in cholangiocytes. Thy-1 positive cells showed a surprisingly low expression of *Mdr1b* mRNA, comparable to resting hepatocytes and cholangiocytes. These results are summarized in Figure 1. The rat liver epithelial cell line RLE φ13, often used as a model for hepatic oval cells, was found to retain the ABC expression profile of oval cells by and large, but did have a high mRNA expression level of *Mdr1b*. Data are interpreted to indicate that hepatocytes are protected by high levels of Mdr1b, while oval cells are protected by high levels of Mrp1 and Mrp3. Via Mrp1, the latter cells are able to efficiently remove products of oxidative stress reactions such as GSSG or the GSH conjugate of 4-hydroxynonenal. High expression of Mrp3, on the other hand, allows for the export of bile salts and glucuronidated compounds.

Hepatic progenitor cells are also activated in various human liver diseases, for instance during viral infections. In **chapter 4**, we studied the expression of ABC transporters in human liver specimens from patient diagnosed with primary biliary cirrhosis, chronic hepatitis C, or submassive liver cell necrosis and compared these with normal liver. By using dilution series of specific antibodies, we were able to quantify the degree of up- or down-regulation of protein expression. In normal liver, hepatocytes and cholangiocytes expressed MDR1. MRP3 was expressed in cholangiocytes and in hepatocytes surrounding the central vein. MRP2 and BSEP expression was, as expected, hepatocyte-specific. There

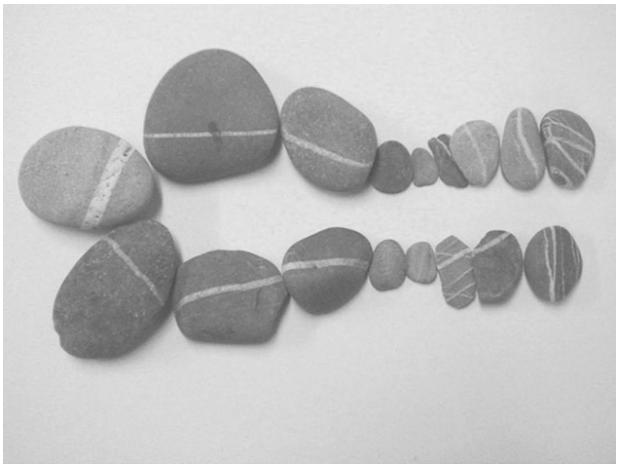
was no detectable expression of MRP1. Under conditions of regeneration after massive hepatocyte loss, remaining hepatocytes expressed high levels of MDR1, MRP1, and MRP3. Expression of MRP2 and BSEP was only decreased in hepatocytes with severe cholate stasis. At the same time, expression of MDR1, MRP1, and MRP3 was highly increased in ductular structures close to the parenchyma. It has been postulated that these ductules form a reservoir in which toxic bile can accumulate. MRP3 may function as a basolateral transporter, extruding bile salts back into the systemic circulation.

A striking difference in ABC transporter gene expression between the rat model described in chapter 3 and the human liver specimens studied in chapter 4 relates to the expression pattern of Mdr1b versus MDR1. Whereas progenitor cells in rats do not show high expression levels of any of the P-glycoproteins, MDR1 is highly expressed in human progenitor cells. This may be related to the severity of liver damage, as the human liver specimens were obtained from patients with end-stage liver disease. In addition, MDR1 levels in humans are in general higher than the Mdr1a/Mdr1b counterparts in laboratory animals.

The most pronounced effect of PHx in rats was on the expression of *Mdr1b* (chapter 2). In **chapter 5**, we aimed to gain insight in the regulatory mechanism involved in this induction. As the cytokine tumor necrosis factor-alpha (TNF-α) has an essential role in liver regeneration after PHx, we hypothesized that *Mdr1b* expression after PHx was, at least in part, induced by TNF-α. We developed an *in vitro* system, using cultured primary rat hepatocytes or a rat hepatoma cell line, in which *Mdr1b* mRNA levels could be induced by TNF-α. We selectively used constructs expressing dominant negative Fas-associated death domain protein (FADD), TNF receptor associated factor-2 (TRAF2) or IκB to inhibit pathways downstream of TNF receptor-1. Inhibition of NF-κB activation prevented induction of *Mdr1b* gene expression by TNF-α, demonstrating the essential role of NF-κB in the induction of *Mdr1b* by TNF-α. Because p53 is up-regulated by TNF-α in an NF-κB-dependent manner and the *Mdr1b* promoter contains a p53 binding site, we used liver cells expressing a dominant negative p53 to show that TNF-α up-regulation of *Mdr1b* is independent of functional p53. Using transient transfection assays we were able to show that *Mdr1b* up-regulation correlated with activation of the promoter. Mutation of the NF-κB site in the *Mdr1b* promoter prevented its induction by TNF-α, showing that activation of the rat *Mdr1b* gene by TNF-α is a direct result of NF-κB binding to the promoter.

From these studies we conclude that there is increased expression of specific ABC transporters in different hepatic cell types during liver regeneration. Proliferating hepatocytes express high levels of Mdr1b. Likewise, hepatocytes in severely damaged livers express Mdr1b/MDR1. Rat hepatic oval cells express high levels of Mrp1 and Mrp3, whereas human progenitor cells express high levels of MDR1, MRP1, and MRP3. We hypothesize that expression of these transporters help these cells to withstand the unfavourable conditions associated with severe liver damage. Understanding the protective function of these transporters, together with elucidation of the regulatory mechanisms involved, may contribute to the development of novel therapies for severe liver diseases.

Samenvatting



De lever heeft een aantal belangrijke functies, waaronder het produceren van gal en het verwerken en afbreken van schadelijke verbindingen. Zij bestaat uit verschillende soorten cellen. De meeste cellen in de lever zijn de zogenaamde levercellen of hepatocyten. Deze cellen zijn in lange rijen gerangschikt. Ze grenzen aan één zijde aan het bloed, deze zijde wordt het sinusoïdale membraan van de cel genoemd. Tussen naast elkaar gelegen hepatocyten vormen zich de galcanaliculi, kleine kanaaltjes waarlangs de gal wordt afgevoerd naar de galkanalen. Het deel van het celmembraan dat aan deze canaliculi grenst, heet ook wel het canaliculaire membraan. De gal wordt vervolgens langs steeds groter wordende galkanalen afgevoerd. Deze galkanalen worden gevormd door de galgangepitheelcellen, of cholangiocyten. Uiteindelijk wordt de gal uitgescheiden in de darm.

Hepatocyten kunnen stoffen uitscheiden in het bloed of in de gal, waarna deze via de urine of de ontlasting het lichaam verlaten. Uitscheiding van stoffen door de lever gebeurt door transporteiwitten. Voor de lever zijn met name leden van de zogenaamde ABC familie van belang. Deze eiwitten transporteren stoffen, zoals galzouten, tegen een concentratie gradiënt in, de cel uit. Op deze wijze wordt de concentratie van mogelijk schadelijke stoffen in de cel laag gehouden. De grote ABC familie is opgedeeld in 7 klassen (A-G). Transporteiwitten van de ABC-B en de ABC-C families zijn onder meer belangrijk voor de vorming van gal.

In de humane lever spelen drie leden van de ABC-B subgroep een belangrijke rol: MDR1 (Mdr1a/1b in knaagdieren), MDR3 (Mdr2 in knaagdieren) en BSEP. MDR1 kan zeer verschillende soorten stoffen transporteren, waaronder verbindingen die slecht in water oplossen, zoals kleine peptiden. MDR3 zorgt voor de uitscheiding van fosfolipiden, vetachtige verbindingen, naar de gal. BSEP is de pomp voor galzouten. Deze eiwitten zijn gelokaliseerd in het canaliculaire membraan van de hepatocyten.

De transport eiwitten MRP1, MRP2, MRP3 en MRP6 behoren tot de ABC-C familie. MRP2 komt tot expressie in het canaliculaire membraan van de hepatocyten. Het transporteert o.a. bilirubine naar de gal. Bilirubine is de stof die bij ophoping in het bloed leidt tot een gele kleur. MRP1 is homoloog aan MRP2, en kan dezelfde substraten transporteren. Het komt in normale lever echter niet tot nauwelijks tot expressie. MRP3 kan ook galzouten transporteren. Van MRP6 is het substraat nog niet bekend. MRP1, MRP3 en MRP6 zitten alle in het sinusoïdale membraan van de hepatocyten. Daarnaast komt MRP3 ook nog in de cholangiocyten voor. Deze transporteiwitten scheiden dus stoffen uit in het bloed. Figuur 2 geeft een schematisch overzicht van de hepatocyten, cholangiocyten en de lokalisatie van deze transporters.

Eerder onderzoek heeft al uitgewezen dat deze transporteiwitten een grote rol kunnen spelen bij het ontstaan van leverziekten. Wanneer bepaalde ABC transporteiwitten afwezig zijn, kan dit tot ernstige problemen leiden. Omgekeerd is ook duidelijk geworden dat van bepaalde transporteiwitten de hoeveelheid toeneemt na beschadiging van de lever, bijvoorbeeld door ziekte, een virus infectie of blootstelling aan giftige stoffen. Dit gebeurt zeer waarschijnlijk om (verdere) schade aan de lever te beperken.

De lever bezit het opmerkelijke vermogen om te delen wanneer er een tekort aan weefsel ontstaat; dit proces wordt ook wel leverregeneratie genoemd. Zo zullen na operatieve verwijdering van grote delen van de lever de overgebleven cellen in de lever gaan delen. Bin-

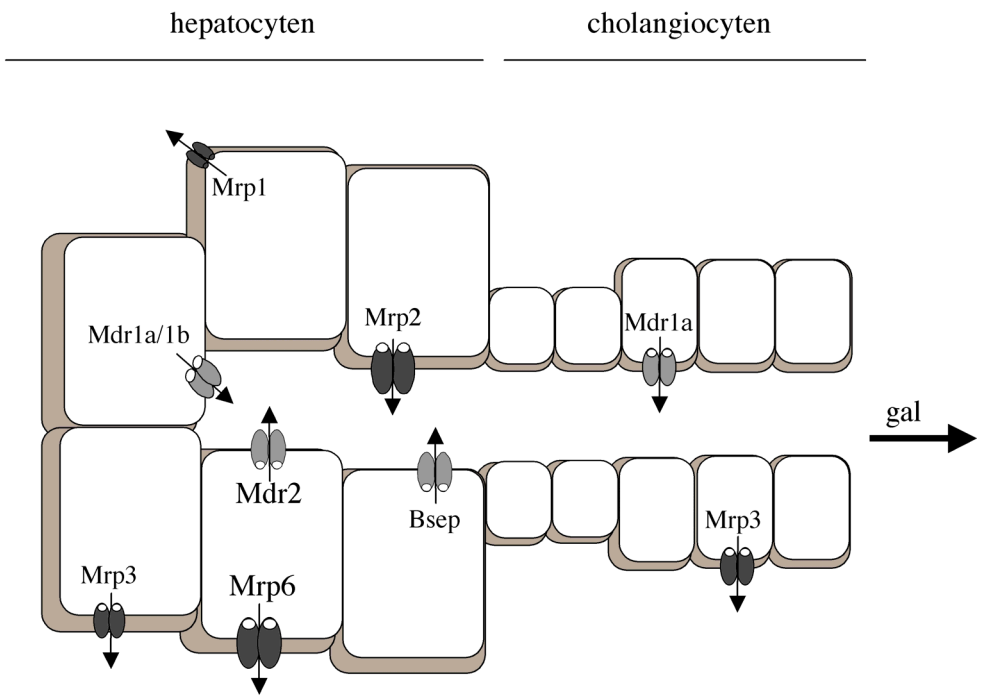


Figure 2: Schematisch overzicht van ABC-B (licht grijs) en ABC-C (donker grijs) transporteiwitten in de lever van een rat.

nen enkele weken heeft de lever zijn oorspronkelijke massa weer terug. Ook tijdens ernstige leveraandoeningen, waarbij cellen in de lever afsterven, kunnen overgebleven cellen in de lever gaan delen om het tekort op te heffen. Naast het herstellen van weefseltekort moeten de overgebleven cellen echter ook de normale functies van de lever uitvoeren. Men kan derhalve veronderstellen dat deze cellen zich aanpassen aan de veranderde omstandigheden en over goede beschermingsmechanismen beschikken, om te kunnen overleven en te kunnen delen. Een hoge expressie van specifieke ABC transporteiwitten zou hierin een rol kunnen spelen. In dit proefschrift is daarom gekeken naar de aanwezigheid van specifieke ABC transporteiwitten tijdens het proces van leverregeneratie.

In **hoofdstuk 1** van dit proefschrift wordt een overzicht van de bekende ABC transporteiwitten gegeven. Tevens wordt er een overzicht gegeven van de verschillende manieren waarop de lever van schade kan herstellen.

In **hoofdstuk 2** is gekeken naar de expressie van ABC transporteiwitten tijdens leverregeneratie in ratten. Hier is operatief ∼70% van de lever verwijderd ("gedeeltelijke verwijdering van de lever" of partile hepatectomie, PHx). Na 24 uur is het resterende deel van de lever uitgenomen en geanalyseerd. De hepatocyten bleken met name een sterk verhoogde expressie van het ABC transporteiwit Mdr1b te hebben. Mogelijkerwijs kunnen levercellen via Mdr1b toxische verbindingen, die tijdens leverschade ontstaan, makkelijk uit de cel naar de gal pompen.

Onder sommige omstandigheden kunnen hepatocyten niet delen, bijvoorbeeld na een

infectie met het hepatitis C virus. Schade aan de lever leidt dan tot de activering van hepatische stamcellen. Deze cellen zijn gelokaliseerd in de overgangen van de hepatocyten naar de kleinste galgangen. Activering van deze stamcellen resulteert in de vorming van de zogenaamde ovale cellen. Deze ovale cellen kunnen vervolgens óf hepatocyten óf cholangiocyten vormen. Zolang bij ernstige leverschade de stamcellen onbeschadigd blijven, kan de lever regenereren. Wij veronderstelden derhalve dat deze cellen over goede beschermingsmechanismen beschikken, zoals een hoge expressie van specifieke ABC transporteiwitten. Tot dusver was er echter weinig bekend over ABC transporteiwitten in ovale cellen.

In **hoofdstuk 3** is, als vervolg op de PHx studie, gekeken naar de expressie van ABC eiwitten in een rat model voor ovale cel activatie. In dit model wordt de celdeling van de hepatocyten geremd door continue toediening van de zeer schadelijke stof 2-acetylaminofluoreen, waarna 70% van de lever operatief verwijderd wordt. Negen dagen na de operatie, wanneer er een maximale hoeveelheid ovale cellen is, was de expressie van Mdr1b in de hepatocyten sterk toegenomen. Er was echter geen sterke expressie van Mdr1b in de ovale cellen. De ovale cellen hadden daarentegen wel een duidelijke expressie van Mrp1 en Mrp3.

Om de ABC transporteiwitten in ovale cellen verder te karakteriseren, zijn geïsoleerde ovale cellen vergeleken met geïsoleerde cholangiocyten en hepatocyten. Ovale cellen bleken een hoge expressie te hebben van *Mrp1* en *Mrp3*, terwijl de expressie van *Mdr2*, *Bsep*, *Mrp2* en *Mrp6* laag was. Deze laatste bleken juist specifiek in hepatocyten tot expressie te komen. Deze resultaten laten zien dat hepatocyten tijdens leverschade mogelijk beschermd worden door een hoge expressie van Mdr1b, terwijl ovale cellen beschermd zouden kunnen zijn door Mrp1 en Mrp3. Via deze transporteiwitten kunnen cellen de schadelijke producten van oxidatieve stress reacties en galzouten uit de cel naar het bloed pompen.

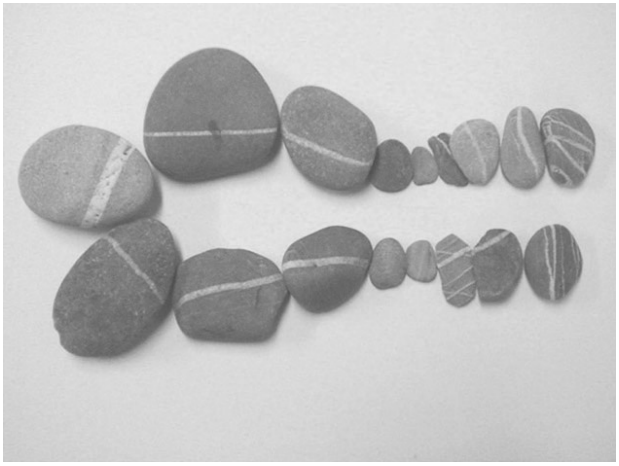
Studies met proefdieren worden natuurlijk gedaan om meer inzicht te krijgen in de werking van het menselijk lichaam. Met de resultaten verkregen in hoofdstuk 3 kon in **hoofdstuk 4** gericht gekeken worden naar de expressie van MDR1, MRP1 en MRP3 in ovale cellen in humane lever. Hiervoor werden leverbiopten gebruikt van patiënten met primaire biliaire cirrose (een ontsteking aan de galgangen), chronische hepatitis C (een virus infectie), of submassieve cel necrose (waarbij grote delen van de lever afsterven). Tijdens al deze ziektebeelden worden de ovale cellen geactiveerd. Onze studie liet zien dat tijdens regeneratie na massale uitval van hepatocyten, de overgebleven hepatocyten een hoge expressie van MDR1, MRP1 en MRP3 hadden. Tegelijkertijd was de expressie van MDR1, MRP1 en MRP3 ook verhoogd in de ovale cellen.

Hoewel de expressie van een aantal transporteiwitten tijdens leverregeneratie nu bekend is, is nog grotendeels onbekend hoe deze veranderingen in expressie gereguleerd worden. Het effect van PHx op Mdr1b expressie in ratten leidde tot de vraag welke factoren in de cel betrokken zijn bij deze reactie. Het eiwit Tumor Necrosis Factor-α (TNF-α) speelt een essentiële rol in het aanzetten van de resterende lever tot groei na PHx. In **hoofdstuk 5** is daarom de invloed van TNF-α op *Mdr1b* expressie onderzocht in gekweekte rat hepatocyten. Hierbij werden routes in de cel, die door TNF-α geactiveerd worden, selectief geremd. Op deze manier werd duidelijk dat de transcriptiefactor NF-κB betrokken was bij de toename van *Mdr1b* door TNF-α.

Uit de resultaten van het onderzoek beschreven in dit proefschrift kan geconcludeerd

worden dat de expressie van specifieke ABC transporteiwitten in verschillende celtypes verhoogd is tijdens leverregeneratie. Delende hepatocyten vertonen een hoge expressie van Mdr1b. Ook hepatocyten in zwaar beschadigde levers hebben een hoge expressie van Mdr1b/MDR1. Ovale cellen in de rat zijn vooral positief voor Mrp1 en Mrp3. In de mens hebben deze cellen naast een hoge expressie van MRP1 en MRP3 ook een hoge expressie van MDR1. Wij veronderstellen dat het tot expressie brengen van deze transporteiwitten de cel kan helpen de nadelige effecten van ernstige leverschade te weerstaan. Verder inzicht in de beschermende rol van deze transporters, tezamen met opheldering van de signaaltransductie routes betrokken in hun regulatie, zal bijdragen aan de ontwikkeling van nieuwe behandelingen voor ernstig leverfalen.

Dankwoord



In juni 1997 kwam ik voor de eerste keer in Groningen...uitgenodigd voor een sollicitatiegesprek. Nu, precies vijf jaar later, is het hele promotieonderzoek afgerond. Dit is gelukt dankzij de hulp van veel mensen, die ik op deze plaats dan ook graag wil bedanken.

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Curriculum vitae

Jenny Ellen Ros werd op 7 april 1973 geboren te Purmerend en groeide op in de Beemster (NH). Na het behalen van het VWO-diploma aan de Rijksscholen Gemeenschap in Purmerend, ging zij in 1991 scheikunde studeren aan de Vrije Universiteit in Amsterdam.

Tijdens haar hoofdvakstage in 1995 heeft zij 6 maanden gewerkt bij de vakgroep Biochemie en Moleculaire Biologie onder leiding van Dr. Harm van Heerikhuizen en Dr. Cindy Gerhardt aan de karakterisering van een G-eiwit gekoppelde receptor in de zoetwaterslak *Lymnaea stagnalis*.

Haar bijvakstage liep zij bij de vakgroep Farmacochemie onder leiding van Prof. dr. Aalt Bast. In het kader van de Erasmus uitwisseling ging zij hiervoor gedurende 6 maanden naar de vakgroep Farmaceutica van de Universiteit van Uppsala in Zweden. Hier werkte zij onder leiding van Prof. dr. Per Artursson en Dr. Katrin Palm aan een model waarmee de invloed van de lading van een molecule op zijn absorptie over het darmepitheel voorspeld zou kunnen worden. Na haar afstuderen in 1996 ontving zij een beurs uit het VSB fonds, die het mogelijk maakte nog 6 maanden bij deze afdeling te blijven werken.

Van september 1997 tot december 2001 was zij als onderzoeker in opleiding (OIO) aangesteld bij de afdeling Maag-, Darm-, en Leverziekten van de faculteit Medische Wetenschappen van de Rijksuniversiteit Groningen op een door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) gefinancierd project. Hier verrichtte zij onder leiding van Prof. dr. Peter Jansen en Prof. dr. Michael Müller het onderzoek dat in dit proefschrift beschreven is. Voor het onderzoek beschreven in hoofdstuk 5 werkte zij enkele maanden op het laboratorium van Prof. dr. Christian Trautwein in Hannover, Duitsland.

Sinds 1 december 2001 is zij aangesteld bij de afdeling Kindergeneeskunde van de Rijksuniversiteit Groningen op een door de Maag Lever Darm Stichting gefinancierd project. Hier doet zij onder leiding van Prof. dr. Folkert Kuipers onderzoek naar factoren betrokken bij de regulatie van de *Mdr2* genexpressie.

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Vos TA, **Ros JE**, Havinga R, Moshage M, Kuipers F, Jansen PLM, and Müller M. Regulation of hepatic transport systems involved in bile secretion during liver regeneration in rats.
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